

Polymers and nanostructured materials for drug nanoparticles, bioimaging, and cell delivery

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Abstract: Polymers can be designed, synthesized, and modified for a wide range of applications. The use of biopolymers and nanostructured materials (either made of polymers or fabricated with polymers) have been extensively investigated for biomedical and biological applications. In this article, we describe the use of polymers for the preparation of poorly water-soluble drug nanoparticles (covering top-down and bottom-up approaches, selection of stabilizers and encapsulation materials), bioimaging (covering polymers, small molecules, nanoparticles for various imaging techniques including x-ray radiography, positron emission tomography, magnetic resonance imaging, ultrasonography, fluorescent imaging, photoacoustic imaging and multimodal imaging), and cell encapsulation and cell delivery (covering the basics, important parameters and applications). We finish the article with perspectives in these exciting research areas. This article is written with the basics and recent progress in the relevant research areas and thus is suitable for a broad readership.

Keywords: biopolymer, drug nanoparticles, bioimaging, cell encapsulation, cell delivery.

1. Introduction

There are many challenges in biomedical research which may be addressed through designing functional biomaterials and new processes. One of the major issues associated with some commercially available drugs, as well as promising candidates, is that they suffer from poor water solubility, leading to low bioabsorption, possible side effects and poor therapeutic efficacy. This problem could potentially be solved by using nanosizing technologies to prepare drug nanoparticle suspensions of poorly water-soluble drugs. These nanoparticles have an increased dissolution rate and improved saturation solubility; however, they usually suffer from poor stability due to high surface energy. In addition to the development of novel fabrication processes, biocompatible polymers are appropriately selected or designed to efficiently stabilise drug nanoparticles; this is essential in the success of such drug formulations, improving the therapeutic effect of many life-changing drugs.

Bioimaging techniques allow researchers and clinicians to non-invasively visualise interior parts of the body in order to study biological processes and help in patient diagnosis and treatment. Many conventional imaging techniques suffer from poor image contrast, low spatial and temporal resolution, and shallow penetration depth. Polymer-based and nanostructured materials have been, and are continuing to be, designed as contrast agents or probes for many different bioimaging techniques. These materials have to be biocompatible for biomedical imaging. Each biomaterial is specifically engineered for a technique and may be further modified for targeted contrast. Improving bioimaging techniques through design of contrast agents will lead to increased early detection of disease and hence improve survival rates.

Cell therapy is an important area of research aiming to provide therapeutic effect by injecting cells into patients, which is often in combination with *in situ* non-invasive bioimaging. Although most of the studies in this area are currently still in research stage, promising results have stemmed from the development of such biomaterials capable of treating a variety of diseases. Continued research in this field could see cell therapy being a viable and effective treatment for many diseases which are currently lacking options.

In this article, we discuss the limitations and shortfalls associated with poorly water-soluble drug nanoparticles, in the context of different fabrication techniques. This is followed by an introduction to commonly used and newly developed bioimaging techniques, with an emphasis on the use and development of contrast agents or probes. Lastly, the use of biopolymers for cell delivery, in conjunction with bioimaging, is discussed. The article ends with conclusions and perspectives in these exciting research areas.

2. Biopolymers for Poorly Water-Soluble Drug Nanoparticles

There are many factors which affect the ability of drugs to carry out their therapeutic effects; for example, poor target specificity, toxicity and low bioavailability. Consequently, many drugs that could potentially treat life-altering diseases do not pass clinical trials or remain in the research stages for an inordinate amount of time. A more contemporary method of drug development, based on high-throughput screening, aims to increase the amount of drugs that make it through clinical trials. The combinatorial chemistry approach utilises vast libraries of compounds to identify lead candidates through screening assays. However, due to the nature of commonly used target-based screening assays, it has also led to an increase in lipophilic drug candidates with low bioavailability, which are therefore not suitable for clinical translation.[1, 2]

As well as potential drug candidates, some clinically available drugs suffer from low bioavailability thus limiting their therapeutic effect when orally administered. Due to their poor solubility, these drugs usually cannot be administered intravenously. A slow dissolution rate means that higher doses are required; however, this may result in toxicity, rendering the drug inadequate.[3] The drug dissolution rate is directly related to water solubility and hence the hydrophilic-lipophilic balance must be considered during oral drug development for adequate absorption in the gastrointestinal tract. Drugs must be highly soluble in water as it is the main component of blood plasma. Therefore, increasing the solubility of poorly water-soluble drugs is of great advantage. However, it is a careful balance as some degree of lipophilicity is required for transport across biological membranes. In 2004, it was found that 17.1% of all World Health Organisation (WHO) essential drugs are classified as BCS II drugs (high permeability and low solubility) and 10.6% as BCS IV drugs (low permeability and low solubility).[4, 5] It is thus clear that there is a high demand for methods to improve the water solubility of drugs.

Nano-sizing drug formulations provide an effective way of improving water solubility. Nanoparticles typically have a particle size of 10-1000 nm, resulting in a high surface area/volume ratio and causing kinetic and thermodynamic characteristics to be altered. Formulating drugs on the nanoscale results in increased solubility as determined by the Ostwald–Freundlich equation (Equation 1),[6] where c_s is the saturation solubility, c_∞ is the solubility of large particles, σ is interfacial tension, V is the atomic volume, R is the gas constant, T is temperature, ρ is the density of the solid and r is the radius.

$$\log \frac{c_s}{c_\infty} = \frac{2\sigma V}{2.303RT\rho r} \quad (1)$$

Additionally, the Noyes-Whitney equation describes how nanosizing results in an increased dissolution rate due to an increased surface area/size ratio as described in Equation 2.[7] D is the diffusion coefficient, A is the surface area of the particle, c_s is the saturation solubility, c_x is the bulk concentration and h is the diffusional distance.

$$\frac{dc}{dt} = D \times A \left(\frac{c_s - c_x}{h} \right) \quad (2)$$

There are many different ways of achieving nanometre-sized drug particles, as described below. After nanosizing, the surface of the resultant nanoparticles can be modified to introduce electrostatic and/or steric groups for stabilisation effects, a process which is necessary for long-term stability and hence good bioavailability.[8] Various biomaterials, typically surfactants or polymers, are utilised to improve stability, but selection is dependent upon the drug nanoparticle surface and the nanosizing technique employed. Nanoparticles are prone to aggregation and Ostwald ripening, which lead to an increase in particle size and particle size distribution.[9] Ostwald ripening occurs when smaller particles dissolve in solution and precipitate onto the surface of larger particles. The effect is caused by the increase in saturation solubility associated with smaller particles, resulting in the migration of smaller particles across a concentration gradient, towards the surface of the larger particles. By adding stabilisers, Ostwald ripening can be prevented resulting in a stable nanosuspension.

As of 2016, there was 51 FDA-approved drug nanoparticles and 77 in clinical trials.[10] This section details various methods of nanoparticle formation as well as a discussion about commonly used stabilisers.

2.1 Top-Down Approaches

Top-down approaches are described as methods which break down large particles into smaller particles through the use of mechanical force. These techniques are used more frequently throughout industry due to simplicity and scalability; however, they are not considered energy or time efficient and it is also difficult to control the particle size distribution.[11]

2.1.1 Milling

Milling techniques are able to reduce particle size by generating shear forces to fracture particles, through collisions with the milling medium.[12] Milling is one of the most commonly used particle size reduction techniques due to high cost efficiency and ease of scale-up. Milling has been investigated for use in other applications including hydrogen storage and solvent-free, sustainable organic chemistry.[13-15]

Dry milling is sometimes employed in the pharmaceutical industry, for example in tableting. The grinding methods can be used on granulated mixtures to ensure narrow particle size distribution of all drug components to prevent segregation in blending.[16] A narrow particle size distribution will lead to high tablet reproducibility and uniformity which is essential when scaling to commercial production. Dry milling is suitable in tableting as the particles are intermediates in the production of pharmaceutical dosage forms. The agglomeration/aggregation problems are minimal because of the presence of stabilizer and the relatively large particles (usually in the micron range). However, in the case of forming drug nanoparticles, due to the high surface area of nanoparticles, more efficient stabilisers must be added to reduce aggregation and improve dissolution.[17] A recent study on dry media milling incorporated polymers using a co-milling method to form a solid dispersion. Probuco^l, a BCS II drug used for the prevention treatment of atherosclerotic cardiovascular diseases, was co-milled alongside a predominantly hydrophilic copolymer (Kolidon VA64[®], poly(vinylpyrrolidone/vinyl acetate), 60/40) to improve dissolution and hence *in vivo* oral bioavailability in rats.[18] The apparent structure transformation of the drug from crystalline to amorphous, through milling, resulted in a drug that is more easily solubilised by polymeric micelles. A homogenous mixture of probu^{co}l/polymer showed a good dissolution rate which was increased upon the addition of a non-ionic surfactant to form a ternary particle. However, with time, the thermodynamically-unstable amorphous particles may convert back to the crystalline form thus contributing to a poor shelf-life, an effect which is exacerbated by the absorption of moisture.[17, 19]

In wet media milling, nanosuspensions are formed when nanoparticles are dispersed in water containing a stabiliser. Wet media milling is already used commercially within the pharmaceutical industry for the production of oral nanosuspensions of Rapamune[®], Emend[®] and Tricor[®] among others.[20] Mechanical forces improve dispersion through exposing the surface area of the nanoparticles to the stabiliser.[21] A schematic representation of wet media milling is shown in Figure 1; the drug suspension is circulated through the milling chamber where shear forces are generated from the milling shaft and nanocrystals are released. The process can be run in recirculation mode to produce nanoparticles of <200 nm after 30-60 minutes of milling.[21]

The final quality of the nanosuspension is dependent on operational parameters such as milling time and speed as well as temperature, bead size and concentration.[22] However, correct selection of one or more stabilisers is believed to be the most important factor for producing a dispersion with long-term stability by preventing Ostwald ripening. There are two types of stabilisation methods: steric stabilisation effects from surface absorption of polymers and electrostatic stabilisation from absorption of ionic surfactants. When selecting a suitable stabiliser, molecular weight of the polymer

must be considered as absorption onto the nanoparticle surface is retarded with higher molecular weight species.[23] Additionally, a high concentration of high molecular weight species would decelerate dissolution in the gastrointestinal tract thus rendering the nanosuspension ineffective. As the stabiliser level must be optimised to improve performance, care must be taken as high concentration of certain polymers, such as sodium lauryl sulphate, can cause unwanted side effects.[23] Currently, there is not a systematic technique used to select stabilisers as it is highly dependent on the surface affinity of the drug and hence a trial and error approach is generally adopted.[24]

Hydroxypropyl cellulose (HPC) is a hydrophilic drug excipient that was shown to stabilise ball-milled Naproxen nanosuspensions with high drug loadings when a secondary stabiliser, arginine chloride, was incorporated.[25] More recently, amino acids have been investigated for their ability to stabilise milled drug nanosuspensions.[26] Amino acids were selected based on binding sites at the drug target, for example, arginine and tyrosine were used for indomethacin, a BCS II non-steroidal anti-inflammatory drug. Vibrational ball milling of drug and the two amino acids in a 1:1:1 ratio co-amorphous blend resulted in superior stability and dissolution properties compared to the free drug. These properties are thought to be due to the increased glass transition temperature (T_g) preventing crystallisation.

Although there is continued interest in researching ways to improve stabiliser selection for this technique, pharmaceutical industries are cautious about the wet media milling method due to contamination issues.[27] Commonly used zirconium oxide beads are prone to abrasion from collision with the milling chamber, resulting in contamination of nanosuspensions.

2.1.2 High Pressure Homogenisation

Another common top-down nanosizing technique is high-pressure homogenisation, which can also be used for cell lysis, emulsification, and processing techniques. It has been used in various industries such as food processing, chemicals and biotechnology.[28] Similarly to milling, high-pressure homogenisation causes particles to fracture, however in this case the shear stress is caused by high pressure.

There are two principle types of high-pressure homogenisation: microfluidisation and piston-gap homogenisation. Microfluidisation works upon the basis of a jet stream, where a nanosuspension is accelerated through a chamber causing particle collision.[29] For Y-type chambers, the nanosuspension is divided into two equal parts and forced to collide. However, for a Z-type chamber, particle collision is due to altering the direction of flow. Piston-gap homogenisation is where a nanosuspension, containing stabiliser, is brought to high pressure (1500 bar) and forced into a narrow gap. The liquid then begins to boil as the vapour pressure is higher than the static pressure which results in cavitation.[30] This phenomenon, along with shear force and particle collision, results in nanosizing of particles.

High-pressure homogenisation has been successfully used in the past to make stable nanosuspensions with an overall drug content of 1-10%. Higher drug loadings are generally more difficult to achieve as the nanosuspension is likely to become viscous resulting in processing difficulties. One review article looked to increase drug loading so that solid drug dispersions could be obtained with less water removal required.[31] A model drug was used where surfactant (Tween 80 and potassium oleate) levels were altered to optimise the nanosuspension. It was found that levels of 0.75% and 1.5%, for Tween 80 and potassium oleate respectively, resulted in the dispersion (20% drug content) with the smallest polydispersity index of 0.24. The homogeniser used could not

process the formulations with a higher drug content, thus demonstrating the limitations of the technique. Larger polydispersity indexes and particle sizes were observed when the surfactant levels were increased or decreased, suggesting reduced stability, thus highlighting the importance of optimising stabiliser levels.

Tween 80 was also used as a stabiliser for nanosuspensions containing an anti-parasitic drug along with co-stabilisers, Poloxamer 188 (a copolymer of polyoxyethylene and polyoxypropylene) and sodium cholate.[32] A stable nanosuspension was made with a good shelf-life, showing potential for oral delivery and improved pharmacokinetics of this poorly soluble drug. Tween 80 (polyoxyethylene-sorbitan-20-monooleate) is a commonly used non-ionic surfactant for drug formulations, as it efficiently solubilises drugs, including various chemotherapeutic agents.[33] However recent research has focused on selecting alternative stabilisers as it has been shown that Tween 80 is pharmacologically active and may generate side effects, such as acute hypersensitivity and peripheral neuropathy in some cases. Similar effects have also been seen for Cremophor EL (polyoxyethyleneglycerol triricinoleate 35), another commonly used non-ionic surfactant.[34]

High-pressure homogenisation was used to form nanosuspensions of Piroxicam, a non-steroidal anti-inflammatory drug, that were consequently lipophilised to form orally disintegrating tablets.[35] In this study, Poloxamer 188 was used as a stabiliser where only a level of 0.5 %w/w was required as the drug content was low (2.5 %w/w). The study highlighted that pre-milling was required to generate micro-sized particles, suggesting that high-pressure homogenisation does not circumvent problems associated with milling techniques, as pre-milling will also lead to contamination issues.

Drug nanosuspensions are an efficient way to tackle the problem of poorly water-soluble drugs. Although top-down methods are used commercially, several issues are associated, as previously discussed. Therefore, investigating the potential of bottom-up approaches as an alternative method for preparing drug nanosuspensions is of high interest.

2.2 Bottom-Up Approaches

Bottom-up methods are used to fabricate drug nanoparticles *via* the assembly of molecules in solution. These methods are not as widely used in industry, compared with top-down approaches, however they possess advantages including better control of crystal growth, resulting in a narrower particle size distribution or smaller nanoparticles. In addition to this, they usually do not require high energy processes or high temperatures. A drawback is the use of organic solvents required for dissolution of the drug, hence limiting use in the pharmaceutical industry so far due to the concern on solvent residual.[36]

2.2.1 Solvent-Antisolvent Precipitation (Nanoprecipitation)

In this method, a water-insoluble drug is dissolved in an organic solvent while stabilisers are dissolved in water, the anti-solvent. When these solutions are mixed, the drug molecules become insoluble and begin to precipitate to form nanoparticles, which are stabilized by surfactant or polymer stabilizer. The technique is dependent on finding a solvent in which the drug will dissolve that is also miscible with the anti-solvent. Hence, ethanol and acetone are commonly used as solvents for when the anti-solvent is water. Precipitation occurs as a consequence of the Marangoni effect, as depicted in Figure 2;[37] a gradient in surface tension will result in the flow of liquid toward the region of high surface tension, resulting in interfacial turbulence.[38] Precipitation is dependent on factors which affect the surface tension, such as temperature, mixing solvent and

stabiliser selection (Figure 2). Polymers, such as poly(lactic-co-glycolic acids) (PLGA), cellulose derivatives or polycaprolactones, can be used as encapsulation materials where particle sizes of 100-300 nm can be achieved with narrow particle size distributions.[39]

Nanoparticle aggregation can occur due to attractive Van der Waal's forces between particles. These attractive forces can be overcome through surface modification of the nanoparticles to create a steric or electrostatic counter-force. In addition to this, nanoparticles are prone to Ostwald ripening;[40] this tends to be a slower process that results in an increase in particle size over longer periods of time. Nanoprecipitation was used for the formation of drug nanoparticles containing paclitaxel, a potent anti-mitotic agent where efficacy is limited due to poor water solubility.[41] This study included PLGA in the solvent phase to encapsulate the drug and Polaxamer 188 as a stabiliser in water. It was found that these nanoparticles showed superior anti-tumour activity compared with free paclitaxel which is thought to be due to the PLGA protecting the drug from hydrolysis and allowing for sustained release over time. Nanoprecipitation has a great advantage in that it requires a one-step synthesis to reproducibly form nanoparticles of 200 nm.

The main advantage of nanoprecipitation is that it does not require mechanical force, as for aforementioned top-down techniques, which requires high energy for operation and may cause contamination. The main drawback to the nanoprecipitation technique is the use of organic solvents. In most cases, there will be residual solvent in the nanosuspension, even after purification. As many organic solvents can have associated hazards, the clinical translation and regulation approval may be hindered.

2.2.2 Supercritical Fluid Precipitation

When a substance is at a temperature and pressure above the critical temperature and pressure, it has the properties of a supercritical fluid. The substance has the density close to a liquid and the diffusability similar to a gas. Supercritical fluids are an excellent solution to purification problems associated with nanoprecipitation as they can be removed by reducing the pressure in the system. Supercritical carbon dioxide (SC-CO₂) is most commonly used, owed to its low toxicity, low cost, inflammability, and relatively mild critical conditions.[42] When applied to nanoprecipitation processes, supercritical fluid can act either as a solvent or an anti-solvent, depending on the techniques employed. For rapid expansion of supercritical solutions (RESS) and rapid expansion of supercritical solutions into a liquid solvent (RESOLV), SC-CO₂ acts as a solvent for hydrophobic drugs. However, it can also act as an anti-solvent in solvent anti-solvent (SAS) precipitation.[43]

More recently, supercritical fluid extraction of emulsions (SFEE) has been used to form stable nanosuspensions, for example with ibuprofen, a poorly water-soluble non-steroidal anti-inflammatory drug. The method involved the formation of an emulsion and subsequent solvent removal by contact with SC-CO₂. [44] Poly(vinyl alcohol) was used as a stabiliser and the particles were functionalised with chitosan to increase the surface zeta potential, thus preventing aggregation. The resultant nanoparticles had a narrow, controllable particle size distribution and were shown to have higher ibuprofen loading compared to an aqueous ibuprofen-saturated solution.

2.2.3 Spray-Drying

Spray-drying is a solvent evaporation technique which is already used throughout the food and pharmaceutical industry. Drug solutions are fed into a chamber and pumped through a nozzle to atomise. The atomised substance is then put in contact with hot air, causing the organic solvent to evaporate and leaving a dry powder which is then separated. The air flow can be either co-current, counter-current or mixed flow, as depicted in Figure 3, depending on the applications.[45] The particle size and shape is dependent on the drying conditions; slow drying will lead to small, dense particles. Also, the Péclet number (Pe), defined as a ratio between drying convection time and the diffusion coefficient of the solid, is associated with the morphology, where small, dense particles are formed at $Pe < 1$. [46]

Spray-drying is a continuous, easily scalable and fast process. However, it may have low cost efficiency and limitations in the case of thermolabile substances. Despite these drawbacks, spray-drying is able to create smaller particles, compared to previously mentioned top-down processes, which are amorphous. In addition to this, an added benefit is that other substances can be easily incorporated through addition into the fluid feed. This has opened pathways to encapsulation of drug particles using spray-drying. Spray-drying is one of the most commonly used techniques to make encapsulated food products, through feeding emulsions into the chamber.[47] Similarly, in the pharmaceutical industry, drugs can be entrapped within the nanoparticles. For example, indomethacin has been encapsulated within poly(caprolactone) nanocapsules using a spray-drying technique where the particles were stable for 5 months.[48] Another study has shown that spray-drying can be used to successfully entrap a model anti-inflammatory drug within chitosan for colonic drug delivery.[49]

2.2.4 Freeze-Drying

Another method of obtaining nanoparticles is by freeze-drying. In this method, a solution is frozen and the solvents are removed through sublimation with low pressures and temperatures. Cryoprotectants and lyoprotectants are often added to the solution to prevent destabilisation due to mechanical stresses incurred in the freezing and freeze-drying stages. Examples of stabilisers include sucrose, trehalose, and poly(ethylene) glycol, among others.[50] Stabilisation at the freeze-drying stage is thought to be due to the stabiliser replacing water molecules lost in the drying process (the water substitute theory) or by removing glassy state molecular motion (vitrification theory).[51]

Freeze-drying of solutions or suspensions is widely used for the production of solid pharmaceutical and particularly biopharmaceutical formulations.[50-52] Emulsion freeze-drying is a technique that has been recently developed to fabricate poorly water-soluble drug nanoparticles.[53] An emulsion is formed when two immiscible liquids are mixed together in the presence of surfactants. When an oil phase is dispersed into an aqueous phase, an oil-in-water emulsion (O/W) is formed or *vice versa*, a water-in-oil emulsion (W/O) can be formed, usually through the use of different surfactants.[54] In emulsion freeze-drying, the poorly water-soluble drug is dissolved in a suitable organic solvent to form the oil phase, which is then emulsified into an aqueous solution containing polymer stabilisers. The resulting O/W emulsion can be directly frozen (usually in liquid nitrogen) and then freeze-dried. During the freeze-drying process, the sublimation of water and organic solvent leads to the formation of drug nanoparticles *in situ* within the porous polymer network, as depicted schematically in Figure 4.[45]

The resultant drug nanoparticles generated through emulsion freeze-drying are highly stable against aggregation due to the surrounding polymer scaffold. Depending on the polymer employed, stabilisation can occur through electrostatic repulsion and/or steric hindrance between nanoparticles. The porosity of the polymer scaffold leads to its fast dissolution in water to release

the hydrophobic drug nanoparticles. The dissolved polymer/surfactant can in turn stabilize the drug nanoparticles, easily achieving a stable aqueous nanoparticle suspension. It is clear there are certain advantages to emulsion freeze-drying compared with other bottom-up techniques.

The success of this technique, in terms of forming stable drug nanoparticles, is dependent upon the properties of the emulsion. For example, the ratio of internal to external phase will alter the size of the oil droplets and influence the concentration and stability of the nanoparticles formed.[55] If the percentage of the internal phase is too high, depending on the type of the surfactants used, it is possible that the emulsion may invert to a W/O emulsion. In addition to this, care should be taken when selecting an organic solvent for the oil phase; it must be immiscible with water but it should also be capable of dissolving the poorly water-soluble drug. More importantly to the freeze-drying process, the melting points of the selected organic solvents should be relatively high (e.g., $> -60^{\circ}\text{C}$) so that the frozen emulsions can be readily freeze-dried.[56] Selection of the stabiliser is critical in forming a stable emulsion. The stabiliser sits at the oil-water interface to reduce surface tension hence it must have hydrophilic and hydrophobic groups to be able to do this. The hydrophilic-lipophilic balance (HLB), an expression which relates to the hydrophilic and hydrophobic groups, can be considered when selecting a surfactant however it has limited use. For example, in many cases polymeric co-surfactants are required to form a stable emulsion. In addition to this, the surfactant level can greatly affect the stability of an emulsion and also the porosity of the polymer scaffold which affects its mechanical stability and dissolution rate in water.

2.2.5 Selection and Design of Stabilisers

Selection of a suitable stabiliser is imperative to prevent particle aggregation. Common surfactants, including ionic surfactants (e.g. sodium dodecyl sulfate (SDS)) and polymeric surfactants (e.g. Tween 80 and Cremophor EL) have been used to stabilize drug nanoparticles. They may, however, induce toxicity or unwanted side effects. Thus, the selection and/or design of biocompatible stabilisers are preferred for pharmaceutical formulations. It is helpful to consider the stabiliser's HLB value and molecular structure in combination with the surface chemistry of the nanoparticle in order to select potentially suitable stabilizers. The performance of the stabilizers will then have to be investigated and demonstrated. **Table 1 summarizes the polymer stabilizers that have been mentioned in section 2 and used for preparation of poorly water soluble drug nanoparticles.**

It should be noted that the HLB values may not be highly indicative of polymer stabilizers. Polymers such as poly(vinyl alcohol) (PVA), polyvinylpyrrolidone (PVP) and more commonly used block copolymers (e.g., the commercially available Pluronic polymers, tocopherol polyethylene glycol succinate) are widely used as stabilizers. In recent years, block copolymer and Miktoarm star polymers have been designed and synthesized as effective stabilizers for nanoprecipitation and forming emulsions.[57]

When preparing drug nanoparticles by emulsion freeze-drying, both polymer (scaffold) and surfactant are usually required to form stable drug nanoparticles with a long shelf-life. For example, Oil Red (OR) nanoparticles, where OR was used as a model and indicative hydrophobic compound, have been formed with both SDS and PVA.[53] In this report, OR was dissolved in cyclohexane and PVA and SDS were dissolved in water and subsequently an O/W emulsion was formed. The emulsion was then freeze-dried to form dry porous composite materials. It was found that high stability was dependent upon inclusion of both PVA and SDS, as a stabiliser and surfactant respectively.[53]

Limitations in reaching higher drug loadings can be a problem associated with the use of both polymer and surfactant in nanoparticle formation. In order to circumvent this issue, it would be ideal to utilise a biocompatible polymer that can act as both a surfactant and scaffold to reduce the formulation complexity. It will also reduce the chance of toxicity and side effects from the stabilizer. As such, a biocompatible and branched block copolymer poly(ethylene glycol)-b-(N-isopropylacrylamide) (PEG-PNIPAM) has been synthesized and used as a single stabilizer to produce poorly water-soluble drug nanoparticles by emulsion freeze-drying.[58] Figure 5 shows the synthesis and structure of the branched block copolymer, where the hydrophobic part (PNIPAM) is lightly crosslinked. When dispersed in water, this branched copolymer can form unimolecular core-shell nanoparticles which can be effectively used to stabilize O/W emulsions.[58] Well-defined nanoparticles were formed *in situ via* the emulsion freeze-drying approach when both a hydrophobic dye and poorly water-soluble drug, indomethacin, were used. Upon stability studies, it was found that the PEG-PNIPAM scaffolds effectively prevented aggregation and formed stable nanosuspensions when dispersed in water. The same branched block copolymer was also successfully used with ibuprofen and ketoprofen highlighting how the block copolymers and methods could be extended to other poorly water-soluble drugs. The results suggest that employing branched block copolymers is an improvement to the already highly impactful technique of emulsion freeze-drying, and imply a great potential in nanomedicine. Furthermore, the unique property of the lightly crosslinked PEG-PNIPAM (i.e. amphiphilic, soluble in polar organic solvent, core-shell nanoparticles in water) also allows the formation of poorly water-soluble drug nanoparticles *via* a simple solvent evaporation approach at room temperature from organic solutions (e.g., with ethanol as the solvent).[59]

2.2.6 Encapsulation Materials

Drug nanoparticles are a general name for nanoparticles that contain drug components. In these nanoparticles, the drug components may be present as molecules embedded in a nanoparticle matrix, as very small particles within micelles, dendrimers or polymer assemblies, or as drug nanocrystals stabilized by polymers or surfactants. Drug nanocrystals are usually prepared by top-down approaches. The emulsion freeze-drying approach may be also used to produce drug nanocrystals.

As previously mentioned, the purpose of preparing poorly water-soluble drug nanoparticles is to enhance dissolution rate and enable aqueous nanoparticle dispersion for direct administration. There are many reports where poorly water-soluble drugs are encapsulated in biocompatible (and biodegradable) polymer nanoparticles to achieve both controlled release and enhanced dissolution in aqueous medium. The encapsulation of poorly water-soluble drugs is usually realized by emulsion evaporation, spray drying, and nanoprecipitation. Generally, in order to encapsulate poorly water-soluble drugs, the drugs and the hydrophobic polymer encapsulating materials are both dissolved in an organic solvent and then processed accordingly. For the emulsion evaporation method, the organic solution is emulsified into aqueous solution in the presence of surfactants or stabilizer. The organic solvent in the emulsion is then removed by evaporation to form nanoparticles. For the nanoprecipitation method, a water-miscible organic solvent is used to dissolve both the hydrophobic drug and the polymer. This organic solution is then mixed with an aqueous solution (usually containing a stabilizer). The nanoparticles may be precipitated during the mixing or after the removal of the organic solvent by evaporation. For the spray drying method, there is no requirement on the solvent's immiscibility or miscibility with water. However, due to the high temperature

involved in the spray-drying chamber, the flammability of the organic solvent must be evaluated carefully. Indeed, the spray drying method is not widely used for organic solutions.

It should be mentioned that hydrophilic drugs, particularly biopharmaceuticals, are also encapsulated in hydrophilic polymer nanospheres (usually crosslinked) to improve the drug stability and achieve controlled release. Some of the most commonly used encapsulation materials are described below:

Poly(lactic-co-glycolic acid) (PLGA). PLGA is a copolymer of lactic acid and glycolic acid monomers, as shown in Figure 6. It is synthesized through a ring opening co-polymerisation reaction with cyclic dimers of the two monomers, where both random and block aliphatic polyesters can be made. Therapeutic devices based upon PLGA have high probability of clinical translation, as it is approved by the USA Food and Drug Administration (FDA) and has excellent biocompatibility and biodegradability.[60, 61] When PLGA is administered into the body, it undergoes hydrolysis *via* cleavage of the ester bond in the presence of water. This produces lactic and glycolic acid which are involved in various metabolic processes and hence show minimal toxicity.

PLGA can be either amorphous or crystalline depending on the block structure and the ratio of lactic to glycolic acid.[62] The crystallinity has a direct effect on the biodegradation rate as it can alter the mechanical strength as well as the ability of the polymer to undergo hydrolysis. In addition to this, factors such as surface modification, preparation method, particle size and drug molecular weight also affect the release properties from within PLGA-based nanoparticles. There are various methods employed to make PLGA nanoparticles, as described in Figure 7, where nanoprecipitation is the most common.[63] Typically, particle sizes of PLGA-based nanoparticles are in the range of 100-250 nm.[64]

Despite the clinical translatability of PLGA-based nanoparticles, there are some disadvantages which have led to the development of nanoparticles using alternative biomaterials. For example, PLGA-based nanoparticles typically have poor drug loading hence large amounts must be administered to have the desired effect. In some cases, this is acceptable due to the inherently low toxicity associated with even high levels of PLGA. However, it often calls for a more efficient route to drug nanoparticles. Another drawback is the fast burst release of drug which may mean that the drug does not reach its target; this effect may be explained by poor encapsulation efficiency resulting in drug molecules loosely attached to the surface.[63]

As previously discussed, paclitaxel is one of the most promising chemotherapeutic drugs.[41] Paclitaxel works by binding to microtubules and preventing the separation of chromosomes during the metaphase part of the cell cycle. However, the effect of paclitaxel is severely hindered by its poor bioavailability. PLGA-based nanoparticles, made by nanoprecipitation, were able to protect paclitaxel resulting in improved potency compared to the free drug.[41] However, it was also noted that there was an initial fast drug release which may have been due to drug molecules that were not sufficiently encapsulated. Another study sought to use a hydrophobic surfactant, 1- α -dipalmitoyl-phosphatidylcholine, and cholesterol to improve encapsulation efficiency of paclitaxel containing PLGA-based nanoparticles by 23%.[65] The aim was also to avoid the use of previously used surfactant, Cremophor EL, which is known to have unpleasant side effects. In this case, microspheres (0.9-3.1 μm) were made using a spray-drying method. A burst release rate was also seen, however in this case it was due to water penetration within the pores of the microsphere.

Poly(lactic Acid) (PLA). PLA is also used to make drug nanoparticles because it is biocompatible and biodegradable. It is hydrolysed into lactic acid upon contact with water. Solvent evaporation methods are commonly used to synthesize PLA nanoparticles, while a salting out technique may also

be employed.[63] The advantage of this method is that it avoids causing stress to the polymeric micelles as the solvent is removed through adding a salting agent such as magnesium chloride.[66]

PLA-based nanoparticles of oridonin, a naturally occurring diterpenoid used to treat oesophageal and hepatic cancer, have been made.[67] PLA-based nanoparticles are less common than those made with PLGA. This is because PLA has a lower molecular weight resulting in an even faster degradation rate so hence sustained release profiles are difficult to achieve.

Chitosan. Chitosan is a polysaccharide consisting of β -(1 \rightarrow 4)-linked D-glucosamine units (Figure 8) derived from the N-deacetylation of chitin, the structural component of crustacean shells. In terms of drug delivery, the presence of primary amine groups renders chitosan superior to other natural biomaterials as it is cationic in neutral or basic conditions, and hence mucoadhesive.[68] The excellent biocompatibility of chitosan also makes it an attractive material for drug encapsulation.

Chitosan nanoparticles are most commonly made using an ionic gelation method, where assembly occurs due to electrostatic attraction between cationic chitosan and a polyanion, such as tripolyphosphate.[69] The simplicity and mild conditions associated with this method mean that other methods such as precipitation and emulsion techniques are disfavoured. Chitosan has been used, alongside alginate, to form nanoparticles containing insulin to improve its oral bioavailability.[70] Insulin is a hormone that regulates many metabolic processes, in particular the conversion of glucose to glycogen. It is administered to sufferers of type I diabetes *via* injection. However, oral administration is desired when frequent doses are required.

Poly(ethylene glycol) (PEG). PEGylating the surface of nanoparticles can increase the bioavailability due to the hydrophilic nature of PEG groups.[71] PEGylated nanoparticles have an increased half-life in the blood stream, as hydrophobic nanoparticles would be recognised by the reticuloendothelial system (RES) and consequently eliminated from the blood stream.[64, 72] In addition to this, PEG groups can also affect drug release properties. An example of this was where progesterone –loaded PLA-PEG-PLA nanoparticles were prepared and it was found that release properties could be controlled by altering the PEG content.[73] PEG also has low antigenicity and immunogenicity and has been approved by the FDA for use as vehicles in food, pharmaceuticals and cosmetics.[74] Other properties include minimal toxicity and good chain mobility, hence highlighting the many advantages of PEGylating nanoparticles.[75]

One of the first clinically available drug treatments containing PEG was approved by the FDA in 1990 for treatment of severe combined immunodeficiency (SCID) due to inherited adenosine deaminase (ADA) deficiency.[76] PEG-modified ADA extended the half-life and hence higher blood levels of the missing enzyme could be achieved. Since then, there has been continued interest in developing PEGylated drug nanoparticles due to the high clinical translatability. For example, PEGylated PLGA vehicles have been investigated for targeted delivery of cisplatin for treatment of prostate cancer.[77]

3. Molecular and Nanostructured Contrast Agents for Bioimaging

Bioimaging techniques are used to visualise interior parts of the body in order to identify abnormalities, tumour growth, poor organ function etc. Bioimaging techniques, particularly non-invasive techniques, are essential for pre-clinical and clinical applications. Some of the most commonly used techniques are illustrated in Figure 9.[78]

The most common clinically available bioimaging techniques are based on radiography, magnetic resonance imaging (MRI), ultrasonography or nuclear imaging. However, each technique has

associated limitations, which may be addressed *via* the development of new bioimaging methods or novel probes to improve sensitivity. The latter is the preferred option as it is a more clinically translatable route and hence this area of research has become intensively investigated. This section will explain the principles behind various bioimaging techniques and current biomaterials being utilised as contrast agents.

3.1 X-Ray Radiography

There are a few techniques that use x-ray radiation (wavelength in the range of 0.01-10 nm) to produce an image. In projectional x-ray radiography, x-rays are produced by an x-ray generator and focused at the part of the body to be imaged. The x-ray is then transmitted through the body and detected by an x-ray sensitive film. An image is then built up based on the amount of x-ray radiation absorbed by the body, which gives information about the composition and density of the specific part of the body imaged.[79]

Projectional radiography is mainly suitable for imaging bones and dense tissues where x-ray absorption is high. However, computed tomography (CT scan) is more commonly used to image both soft and hard tissues by using higher energy radiation. In this case, the x-ray sensitive film is replaced by sensitive scintillation detectors giving much higher contrast between different densities.[80] Tomographic images are produced through the computer-processing of x-ray radiation exposure at different angles. CT scanning has allowed for much more effective diagnosis of cancers and lesions.

One of the main limitations of using x-ray radiation is poor contrast; it also has associated health risks. X-ray radiation is ionising and can interact with water molecules to generate hydroxyl free radicals.[81] These species cause damage to nearby DNA which can result in mutations that lead to cancer. There is therefore a limit on the amount of CT scans given to a patient or animal and it is not considered appropriate for pregnant women.

3.1.1 Radiocontrast Agents

Radiocontrast agents are administered to patients to enhance contrast in x-ray examinations. They are predominantly based on iodine or barium compounds which have high attenuation in the x-ray region of the electromagnetic spectrum.

3.1.1.1 Iodinated Contrast Agents (ICA)

ICAs have been in use since the 1950s and are the most commonly administered radiocontrast agents.[82] There are ionic or non-ionic ICAs available. Non-ionic, organic based ICAs are used at present due to slightly reduced side effects. The structure of non-ionic ICAs is based upon a 2,4,6-triiodinated benzene ring and it is the atomic radius of iodine which results in x-ray attenuation. The biomaterial is designed so that there are three iodine atoms in close proximity for maximum attenuation. However, the inclusion of a stable benzene ring prevents the release of toxic iodide ions, hence showing a benefit over ionic ICAs. The other positions at the benzene ring can be functionalised to improve properties such as solubility and toxicity.[83]

Although ICAs are frequently used, there are associated unpleasant side effects, including nausea and vomiting (usually within 24h) which affect a small group of patients. More serious side effects have also been observed where symptoms appear after a certain amount of time. The main problem of this type is contrast-induced nephropathy (CIN), which can lead to acute renal dysfunction.[84] High-risk patients include those with a pre-existing kidney problem, anaemia or diabetes mellitus, as well as elderly patients. It is possible to reduce the adverse effects by ensuring the patient is well hydrated. However, it is not always effective in such high-risk patients and hence sometimes dialysis

may be required and mortalities can occur.[85] Some researchers are developing methods to reduce the effects of CIN, for example with antioxidants or HMG Co-A reductase inhibitors, however limiting doses is advised.[86]

3.1.1.2 Barium Sulfate

Barium sulfate is a contrast agent used to image the gastrointestinal (GI) tract. It is a white powder which can be made into a slurry and administered orally to provide high x-ray attenuation in order to provide information about digestive problems. For example, it has been used for detecting problems with the oesophagus, such as post-operative oesophageal leaks or perforations.[87, 88] Although it is relatively safe and non-toxic, the use of barium sulphate is limited to the GI tract.

3.2 Positron Emission Tomography (PET)

PET is a clinical and preclinical imaging technique which detects biological molecules that have been labelled with positron-emitting radionucleotides. During a PET scan, a radioactively labelled biological molecule is administered to patients, which has usually been designed so that it will target a specific area of interest. The fundamental idea is that the radionucleotide will decay leading to positron emission with time. The positron, also known as an antielectron, will travel to the tissues and interact with an electron in a process known as annihilation. This consequently produces a pair of gamma rays which will travel in opposite directions towards the detectors.[89, 90]

PET is a technique with high sensitivity, especially compared to single photon emission computed tomography (SPECT), and can generate 3-dimensional images.[90] This high sensitivity means that only small amounts of contrast agent are needed. Despite these advantages, PET also utilises ionising radiation and hence use is limited, because over-exposure has associated safety risks.

3.2.1 Radionucleotide Tracers

3.2.1.1 Fludeoxyglucose (^{18}F FDG)

^{18}F FDG (2-deoxy-2- (^{18}F) fluoro-D-glucose) is the most commonly used PET tracer (Figure 10). It is usually administered intravenously, 50 minutes prior to carrying out the scan to allow time for uptake by tissues.[91]

As ^{18}F FDG is chemically similar to glucose, PET can be used to assess tissue metabolism by monitoring ^{18}F FDG uptake by cells. Hence, PET can be used to detect some cancers by monitoring metabolic abnormalities associated with tumours.[92] Detection efficiency is affected by many factors including tumour size, metabolic activity and serum glucose level. In addition to this, any surrounding background activity can have an effect. Therefore, there are cases where false negative results can occur, for example with diabetics, who have unregulated serum glucose levels.

3.2.1.2 ^{89}Zr Zirconium

^{89}Zr Zirconium is a relatively new radioisotope being considered for use in PET scans which has shown promising results in preclinical models. One of the main advantages of zirconium compared to ^{18}F FDG is an extended half-life, meaning that repeated scans can be taken over several days. The half-life is 110 minutes and 78.4 hours for ^{18}F and ^{89}Zr respectively.[93]

Despite the clinical availability and high sensitivity of PET scans, there are still some limitations. Aside from the health risks of ionising radiation, another drawback is the poor spatial resolution of only a few millimetres. Recent work has looked at designing hybrid contrast agents to combine the high spatial resolution of magnetic resonance imaging with the high sensitivity of PET.[94]

3.3 Magnetic Resonance Imaging (MRI)

MRI is an attractive technique as it has very limited health risks because it does not utilise ionising radiation. It is the most commonly employed technique for brain imaging. The principles behind MRI exploit the relaxation properties of water protons in the body.[95] A patient is put inside a metal coil which has a strong magnetic field; this causes water proton alignment within the direction of the magnetic field which is disturbed upon introduction of a perpendicular radiofrequency pulse.[96] Once this is removed, protons returned to their aligned state within the magnetic field and this process is known as relaxation. Protons in different organs/tissues/fats relax to different extents and result in the complex greyscale images produced.

There are two types of magnetic relaxation: longitudinal (T_1) and transverse (T_2).[97] A third relaxation time, single T_2 decay time T_2^* , is also used. T_1 is also known as spin-lattice relaxation and describes the relaxation of a proton from a state perpendicular to the direction of the magnetic field. It is affected by the local magnetic fields of nearby nuclei. T_2 , also known as spin-spin relaxation, is the loss of coherence in an ensemble of nuclei spin. Different contrast agents can be administered to patients to accelerate either T_1 or T_2 relaxation rate in certain tissues and thus improve image contrast.[98]

The ability of these materials to improve contrast can be expressed by relaxivity (r_1 , r_2), a term which relates the proton relaxation rate (T_1 , T_2) to the concentration of contrast agent (CA), as shown in Equation 3.[99]

$$r_1 = \frac{1/T_1}{[CA]}, \quad r_2 = \frac{1/T_2}{[CA]} \quad (3)$$

Some of the commonly used MRI probes are described below.

3.3.1 Gadolinium-based Contrast Agents

Contrast agents based on gadolinium(III) ions are commonly used for accelerating T_1 relaxation rate. Gadolinium(III) is a paramagnetic lanthanide species with 7 unpaired electrons and it is preferred over other elements, such as dysprosium(III) and holmium(III), due to its symmetrical s-state.[99] Gadolinium alters relaxation properties through an interaction with local water protons. The mechanism is thought to involve changes in the water exchange rate in the gadolinium coordination sphere and water diffusional correlation time, as described by the Solomon-Bloembergen-Morgan (SBM) theory.[100, 101]

Elemental gadolinium(III) is toxic and hence chelation is essential before administration to patients. Organic chelators are often used, where macrocyclic, multidentate compounds are preferred to their linear analogues, in order to reduce transmetallation through stronger binding.[102] Many clinically available gadolinium-based contrast agents contain macrocyclic chelators, for example Dotarem® depicted in Figure 11a. Gadolinium species have shown to be significantly less nephrotoxic when compared with ICAs, even at high doses.[103] Despite this, there is evidence to show that gadolinium-based contrast agents can be linked to nephrogenic systemic fibrosis (NSF) and hence the FDA has advised that, if possible, clinicians should avoid using some contrast agents such as Magnevist® shown in Figure 11b.[104]

One of the limitations of gadolinium-based contrast agents is their low sensitivity, and hence, recent research has focused on ways to improve this. One way of doing this is by linking multiple gadolinium complexes together in order to increase the rotational correlation time, as demonstrated

in Figure 12.[105] Rotational flexibility also affects relaxivity, where greater enhancement is seen with dendrimers (Figure 12b) compared to linear oligomers (Figure 12a). However, optimum enhancement is achieved when gadolinium is at the barycentre (Figure 12c).

Another way of boosting relaxivity values is by confining gadolinium chelate species within porous materials.[106] This effect has shown to improve image contrast through restricted tumbling of the gadolinium chelate combined with a slower diffusional correlation time associated with the water molecules.[107] Porous silica nanoparticles, metal-organic-frameworks and polymer based materials are currently being investigated for their ability to improve image contrast through immobilisation of gadolinium chelate species.[108] Recently, albumin-based nanoparticles were synthesized and showed superior relaxivity values compared with other clinically available gadolinium-based contrast agents.[109]

This idea of immobilisation of contrast agents within porous networks opens a door to multifunctional imaging whereby the external surface of the porous molecule may be easily functionalised for targeting or drug delivery purposes. For example, the surface of mesoporous silica nanoparticles (MSNs) can be modified with folic acid in order to target tumours.[110] This idea could be applied in MRI to selectively provide contrast to specific parts of the body. In addition to this, MSNs can be functionalised to allow the holding and release of drug molecules.[111] This effect, combined with the ability to immobilise gadolinium chelate species within MSNs, could provide a novel theranostic probe for simultaneous diagnostic and therapeutic capabilities.[112]

3.3.2 Superparamagnetic Iron Oxide (SPIO)

SPIO or superparamagnetic iron oxide nanoparticles (SPIONs) exist as colloidal suspensions of magnetite (Fe_3O_4) or maghemite ($\gamma\text{-Fe}_2\text{O}_3$), which exhibit high magnetism upon introduction of an external magnetic field.[113] They predominantly provide contrast by altering the T_2 relaxation rate.[114] The crystal structure of the iron oxides is part of the spinel group and the magnetism in magnetite occurs due to the electronic exchange between Fe(II) and Fe(III) sites. SPIONs are ideal for biomedical applications due to their low toxicity, excellent colloidal stability and ease of surface modification.[115] They are metabolised and become a source of iron for the body, where they are eventually incorporated into red blood cell as haemoglobin.[116] Aside from applications in MRI, they have also been used in the chemical and environmental industries, for example in water treatment.[117] In the medical sector, their high magnetism can also be exploited for therapeutic applications such as magnetic targeted drug delivery.[118] Examples of previously used commercially available SPIONs for MRI include Lumerin® for imaging of the gastrointestinal tract and Endorem® for the liver/spleen.[119]

SPIONs are usually made *via* a wet chemical route involving co-precipitation of Fe(II) and Fe(III) salts with a base such as ammonium hydroxide in aqueous solution.[120, 121] By altering the ratio of Fe(II) and Fe(III), the shape, size and composition of the nanoparticles can be controlled.[115] Additionally, the type of salt, pH and ionic strength can alter the final properties.

SPIONs are relatively stable due to the high magnetism, however the small size of the particles results in high surface energy which can cause aggregation. Consequently, surface modifications are introduced to improve the stability of the suspension. Polymeric coatings are often employed where molecular weight, chain length, attachment method, surface coverage can affect the properties of the resultant nanoparticles.[122] These parameters can be tailored to alter hydrophilicity, biodegradation, bioavailability etc., and consequently the performance of the SPION.

Core-shell encapsulation is another approach to surface functionalisation of SPIONs. Inorganic materials such as gold or silica can effectively encapsulate SPIONs, providing protection and inertness. SPIONs with an average particle size of 13 nm have been synthesized with a porous silica coating of approximately 2 nm in thickness.[123] Naproxen, a non-steroidal anti-inflammatory drug, was incorporated into the silica network, where release was observed upon dispersion of the nanoparticles. The silica coating and drug entrapment did not deteriorate the magnetic properties of the SPIONs suggesting that they could still be used as contrast agents. This study demonstrated the great potential for using SPIONs for simultaneous drug delivery and MRI.

The surface of SPIONs can also be functionalised in order to improve bioavailability or biocompatibility.[124] As previously mentioned, PEG is commonly used to increase the half-life of nanoparticles in the blood as it is hydrophilic and less likely to be eliminated by the RES.[64] Another example is where pullulan-coated SPIONs showed improved cellular uptake and reduced cytotoxicity compared with non-functionalised SPIONs.[125] Functional ligands can also be attached to the SPION to introduce benefits such as therapeutic release and targeting, as depicted in Figure 13.[122]

3.4 Ultrasonography

Medical ultrasonography is most commonly associated with imaging fetuses at regular intervals during pregnancy. However, it can also be used to image the heart, muscles, abdomen etc. Ultrasonography uses sound waves to generate a 3-dimensional image through detecting the reflection/attenuation/scattering of the waves by tissues.[126] It does not use ionising radiation so has minimal risk associated. One of the main drawbacks is that ultrasound relies upon movement in tissues and hence is not able to be used to image tumours. It also has limited depth penetration and is only able to image soft tissue.[127]

Microbubbles have been used to enhance ultrasonic imaging.[128] Microbubbles were first observed in the 1960's by a cardiologist named Dr Charles Joiner.[129] When performing an echocardiogram, he noticed a significant improvement in image contrast after the injection of indocyanine green, which was later discovered to be due to small bubbles forming at the catheter tip. After this, much research was devoted to developing ways to improving the efficacy of microbubbles by stabilising the gas-liquid interface with surfactants, as well as designing targeted microbubbles.[130]

Without a stabiliser, a microbubble will collapse due to high surface tension at the gas-liquid interface. Encapsulation of the microbubble can increase stability by reducing surface tension and preventing air from leaving the inside of the core.[131] Lipids and proteins have been used as materials for encapsulation of microbubbles.[132]

Levovist® is a widely used contrast agent for ultrasonography which contains galactose (99.9%) and palmitic acid (0.1%).[133] The galactose microcrystals are available as a white powder which form a suspension upon addition of water. In suspension, air microbubbles adhere to the surface of the solid galactose microcrystals. This is administered intravenously to patients before ultrasound examinations causing the galactose crystals to dissolve and release air microbubbles into the blood.[129]

3.5 Fluorescent Imaging

Fluorescent imaging techniques are a form of optical imaging, whereby a substance emits light upon absorption of electromagnetic radiation. The principles behind fluorescence can be fully understood by considering the electronic states of the fluorophore. Upon absorption of light, an electron is briefly excited to a higher energy level and a photon is then emitted once this electron returns to its ground state.[134] The photon emitted is usually lower in energy and the difference between the absorption and emission wavelengths is described as the Stokes shift. The efficiency of a fluorophore is described by quantum yield (Φ), as described in Equation 4.[135]

$$\Phi = \frac{\text{photons emitted}}{\text{photons absorbed}} \quad (4)$$

One of the key advantages of fluorescent imaging is relatively high contrast as many cellular components do not fluoresce upon absorption of certain wavelengths of light, for example the near-infrared region.[136] Hence, proteins and biological processes can be visualised through labelling with specific fluorophores. Additionally, fluorophores can be used to label antibodies which selectively bind to target molecules of interest.

Despite the many advantages of fluorescence imaging techniques, the main drawback is limited clinical availability as it is not useful for imaging of intact animals/humans. In practice, it can be used for imaging cell cultures or tissue samples for applications such as immunostaining assays, however whole body fluorescence imaging in humans is highly challenging (there are reports of whole body fluorescence imaging of mice) as signal is attenuated with increasing depth.[137] In the clinic, near-infrared fluorescence can be used during surgery for the detection of sentinel nodes around a tumour.[138] Another disadvantage of fluorescence imaging, along with many other optical imaging techniques, is that it suffers from poor spatial resolution. Discussed below are the various probes or agents that can be used to enhance contrast in fluorescent imaging.

3.5.1 Organic Dyes

Some small molecules are able to fluoresce and can be used for fluorescence imaging. One well-known example is fluorescein, shown in Figure 14a, which can be used in fluorescein angiography.[139] It has an absorption wavelength at 470-490 nm and emission at 520-530 nm. It can be incorporated into a probe for *in situ* hybridisation,[140] a technique used to identify chromosomal abnormalities and hence diagnose genetic disorders, such as leukaemia and autism.

Fluorescein isothiocyanate (FITC), depicted in Figure 14b, is a derivative of fluorescein which has been widely used. One of the main disadvantages of some fluorophores, including fluorescein derivatives, is that they are prone to photobleaching.[141] This is where a photochemical reaction occurs which reduces the ability of the fluorophore to fluoresce.

3.5.2 Quantum Dots

Quantum dots are semiconducting nanoparticles. Their properties, as a result of quantum confinement, are dependent upon particle size, shape, and surface chemistry.[142] The main advantages of quantum dots over organic molecules include resistance to photobleaching, high molar extinction coefficient, broad spectrum absorbance, and high resistance to degradation.[143] The ability to tune absorbance properties through modifying the particle size and shape is probably the most notable benefit.

Currently, CdSe, CdTe, InP, and InGaP are the most common quantum dot materials. Other materials have also been investigated but it is difficult to achieve stable particles with a narrow particle size distribution.[144] A solution to this problem is finding suitable materials to coat the particles, for example some quantum dots can be core-shell particles with a ZnS coating.[143] Work has been carried out to increase the circulation lifetime of core-shell CdSe-ZnS quantum dots for whole body fluorescence imaging on mice.[145] This was done by surface modification, where increased circulation time and reduced non-specific cellular uptake was found with quantum dots modified with long PEG chains.

3.5.3 Nanodiamonds

Diamond is made up of sp^3 hybridized carbon atoms with tetrahedral geometry. All four of carbon's valence electrons in diamond are used up in bonding. Diamond is thus extremely inert, which can be highly beneficial for biomedical applications. Hydrogen or hydroxyl groups on diamond surface can be used to terminate dangling bonds and to increase inertness. Nanodiamonds are nanosized diamond, which can be made by chemical vapour deposition, high-pressure high-temperature or detonation depending on the application.[146]

Synthetic nanodiamonds can be engineered to include nitrogen-vacancy (N-V) defects and so have the properties of semiconductors.[146] Such nanodiamonds have absorbance and emission wavelengths of ~560 nm and ~700 nm, respectively. Their fluorescence is well-separated from the autofluorescence of some cellular components and hence background signal would be reduced compared to some organic dyes, which have shorter wavelength emission. This high contrast coupled with high quantum yield makes nanodiamonds ideal for fluorescence contrast agents both *in vitro* and *in vivo*.

3.6 Bioluminescent Imaging

Bioluminescence imaging is the detection of light which has been biochemically emitted from living organisms. It is particularly useful for imaging mammalian tissues as they tend to have low bioluminescence allowing for a low signal-to-noise ratio and hence high sensitivity.[147] However, one of the main drawbacks of the technique, which thusly inhibits the use on humans, is the need for a substrate such as luciferin.[148] In addition to this, there are issues of signal attenuation with increasing depth.

3.6.1 Luciferase

Luciferase is the general name for a group of enzymes which can emit a bioluminescent signal upon oxidation of a substrate, luciferin, in the presence of oxygen and ATP.[149] The enzyme can be found in a variety of organisms including the North American firefly (*Photinus pyralis*) as well as in certain bacteria, insects and fish.[150] Live animal imaging can be non-invasively carried out by engineering certain cells to express luciferase. It has been investigated for use in cell tracking of stem cells, immune cells and bacteria in rodent models.[151]

3.7 Photoacoustic Imaging

Photoacoustic (PA) imaging is a relatively new technique which combines the advantages of optical imaging and ultrasonography. It works upon the principle that optical absorption of photons, by tissues or PA probes, is converted into ultrasonic waves by the photoacoustic effect.[152] Firstly, irradiation of a light absorbing species generates heat, which causes a rise in the transient local temperature. This leads to thermo-elastic expansion thus producing sound waves that can be detected by an ultrasound transducer. It is currently being trialled in the clinic and there are many

promising studies which suggest that converting optical energy into ultrasound waves has many advantages.[153, 154] Compared with conventional optical imaging and ultrasound, the technique offers benefits such as improved spatial resolution and imaging depth.[155]

Optical microscopy techniques are usually limited by the mean free path of a photon, which describes the distance the photon travels between two scattering events, which in turn leads to a loss of original propagation direction and causes images to blur.[156] Less scattering is seen for near infrared (NIR) radiation (700-1000 nm) compared with the UV and visible spectral region which is dictated by the interaction of the photons with tissues at these wavelengths. Therefore, contrast agents that absorb in the NIR region are ideal for PA imaging; such species allow deep light penetration as tissues have relatively low attenuation of NIR radiation. Some endogenous NIR-active compounds can also be visualised with excellent contrast. For example, haemoglobin has broad spectral absorption and hence PA imaging can be used to image blood vessels.[157]

There are two types of nanoparticles that can be used as exogenous NIR-absorbing contrast agents for PA imaging.[158] The first is nanoparticles with surface plasmon resonance (SPR), whereby optical absorption is dependent on the physical dimensions of the material, for example, gold nanorods. The second type of contrast agents involves light absorbing organic dyes, such as indocyanine green.

Multispectral optoacoustic tomography (MSOT) is a PA imaging technique that utilizes multiple excitation wavelengths to allow simultaneous identification of different light absorbers in tissue.[159] Compared to conventional PA imaging, MSOT is capable of imaging at multiple wavelengths and the information obtained from multiple wavelengths improves image accuracy as a function of penetration depth.[156, 160] Furthermore, multiple absorbers may be employed and the signals generated from multiple wavelengths can be spectrally unmixed. This allows the identification of these absorbers and provides detailed information about the imaged subject.[161] Due to the combined high spatial resolution and deep light penetration, MSOT can be used in real time to monitor pharmacokinetics and biodistribution in organs,[162] enabling both cell tracking and efficacy studies.[163]

More recently, research has focused on combining PA imaging with therapeutic techniques such as photothermal therapy (PTT).[164, 165] This opens an exciting avenue to achieving a novel theranostic tool, combining therapeutic and diagnostic capabilities. An example of this was where gold nanorods were used for simultaneous molecular imaging and selective phototherapy of cancer cells.[166] This idea is realised as the principle theory behind PTT is the photoacoustic effect, where electromagnetic radiation, such as visible light or NIR, is converted to heat in order to kill target cells.

There are many types of nanostructures and dyes which have been used as PA probes for PA imaging or MSOT imaging. The commonly used PA contrast agents are introduced below.

3.7.1 Gold Nanoparticles

Gold nanoparticles in aqueous suspension are usually red owing to their localised SPR, which describes the collective oscillation of conduction electrons when photons are absorbed. The colour can change depending on the size and shape of gold nanoparticles. Gold nanoparticles, including nanoshells, nanorods and nanocages, can be exploited for their SPR properties in order to provide contrast in PA imaging.[165] Modifications in the size and the shape of such nanoparticles causes a shift in the SPR, which in turn alters the absorption properties.[167] This unique phenomenon is very attractive as it allows for the ability to tune absorption properties. Figure 15 shows how the absorbance changes upon modification of the physical properties of the nanoparticles.[168] Another

advantage is that modification of gold surfaces is well-established allowing for targeted contrast or the conjugation of contrast agents for other imaging modalities. It has been found that the most effective way of stabilising gold nanoparticles is by forming a strong Au-S bond where functional thiolates can be introduced to aid stabilisation, for example PEG.[169]

The SPR of gold nanoparticles can be exploited in biosensing applications.[170] The SPR is greatly affected by the chemical environment and hence local changes and attached molecules can be detected by observing shifts in absorbance.[171] Also, some biological moieties can cause aggregation of gold nanoparticles which in turn results in an SPR shift, hence providing a calorimetric detection method for such moieties.[169] For example, a study has shown that this property can be utilized for selective detection of cancer using PA imaging.[172] Conjugation of specific antibodies to gold nanoparticle surfaces results in the aggregation of the probes when bound to epidermal growth factor receptors, an effect which is visible by PA techniques.

Gold nanoparticles are used for other biomedical applications such as in gene and drug delivery [173] as well as in conventional optical or fluorescence imaging, when functionalised with a fluorophore. They show good biocompatibility and minimal toxicity and hence can be easily translated to PA imaging.

Gold nanoshells consist of a dielectric silica core surrounded by a conductive, gold shell.[174] The absorption and scattering properties can be altered by changing the size of the core, as well as the shell. This results in the ability to tune absorption across the visible and infrared regions of the electromagnetic spectrum and in particular the NIR region, where absorption by tissues is minimal. Relative to organic dyes, gold nanoshells are also optically robust. Gold nanorods are another class of colloidal gold which can provide contrast in PA imaging. They are cylindrical in shape and hence changes in symmetry alter SPR absorbance. Gold nanorods are a more recent development in the class of gold nanoparticles as they can be made much smaller than nanoshells. Gold nanocages are porous structures of cuboidal symmetry and have also been used in PA imaging applications.[175] Gold nanocages have demonstrated greater contrast enhancement due to possessing a larger absorption cross-section.[176] This property also makes gold nanocages ideal for photothermal therapeutic therapy, where the structures could be engineered to target cancer cells and cause transient local heating, and consequently cell death, upon NIR irradiation.[177]

Recently, gold-plated carbon nanotubes (CNT) have been shown to be effective in providing contrast in PA imaging.[178] Single-walled CNTs were made with a diameter of 1.5-2 nm and coated with a thin gold layer of 4-8 nm in thickness, where changes in these dimensions altered the absorbance properties. As well as the tunability aspect, another advantage is derived from the large surface area of CNTs for conjugation of molecules.

3.7.2 Organic Dyes

3.7.2.1 Indocyanine Green (ICG)

ICG, depicted in Figure 16, is a cyanine dye used in medical diagnostics. Other cyanine dyes exist, with varying lengths of the polymethine bridge. However, many of them only show absorption in the visible region.[179] As well as fluorescent properties, ICG has peak absorption in the NIR region at 800 nm. It is thus an ideal contrast agent for PA imaging.[180] ICG has been approved by the FDA for over 50 years for medical imaging techniques, such as angiography.[181]

Interesting to note, the fluorescence signal of ICG is remarkably decreased upon protein binding. This characteristic has been exploited for tumour detection. An example of this is where monoclonal antibodies were attached to ICG, which specifically target cancer cell-surface markers, such as anti-CD25, anti-EGFR1 and anti-HER2.[182] This probe was shown to be inactive until application of sodium dodecyl sulphate and 2-mercapto ethanol when a fluorescence signal was observed, thus providing a way of labelling tumours using ICG.

There are certain drawbacks associated with ICG which include short half-life, poor photostability, and low quantum yield. However, there have been recent efforts to circumvent these limitations. Modification of ICG is a highly attractive idea, as such biomaterials would have increased likelihood of clinical translation due to the FDA approval and widespread use of ICG.[165, 183] Photonic explorers for biomedical use by biologically localized embedding (PEBBLE) technology is a way of tackling some of the problems that limit the use of ICG.[184]

PEBBLE technology uses nanoparticles which can incorporate sensor dyes, contrast agents, drugs, photosensitizers and cell-targeting moieties.[165] Encapsulation increases the stability and circulation time of such molecules within the body, a problem which limits the use of the free ICG dye in imaging techniques. In addition to this, the surface of the nanoparticle can be functionalised to improve selectivity of image enhancement. PEBBLES containing ICG have been investigated for imaging as well as for cancer therapy. This is because ICG is also a photosensitiser, producing highly reactive singlet oxygen to kill cells upon light activation. This possibility of combining photoacoustic imaging with photothermal therapy would provide a novel, theranostic tool capable of tumour detection and treatment. The possibility of including a sensor dye within the nanoparticles could allow for functional tracking of biological parameters (e.g. tissue pH, cell membrane potential etc.) using PA imaging. An example of this was where ICG was embedded within an organically modified silica matrix with surface conjugated monoclonal antibodies, specific for prostate cancer targets.[184]

3.7.2.2 Perylene Diimide Derivatives

Perylene-3,4,9,10-tetracarboxylic diimide (PDI) derivatives are characterised by their perylene core and imide groups at either end, as depicted in Figure 17. Advantages of such organic molecules include exceptionally high stability, ease of modification, and low cost. The optical absorption properties can be tailored by altering the size of the aromatic core, where a bathochromic shift to the IR region is seen upon expansion of the core.[185] Additionally, adding substituents to the bay position (R_1) alters the absorption wavelength.[186] Long alkyl-chains can be added to the imide position (R_2) to improve the solubility of PDIs; this modification does not affect the electronic properties as there are molecular orbital nodes at this position. Hence, it is clear that PDI derivatives are highly versatile molecules that can be tuned for various applications, such as PA imaging. PDI derivatives are also utilised as n-type semiconductors in optoelectronics and photovoltaics.[187]

PDI can be modified to attain NIR absorbance for PA imaging by adding substituents, such as amines, to the bay position. However, one of the main limitations of PDI is poor water solubility, which is thus hindering clinical translation. As mentioned in section 2, one way to circumvent the poor water solubility is by forming nanoparticles. Therefore, many of the methods of nanosizing drug therapies could, in theory, be applied to PDI.

Recently, PDI nanoparticles have been synthesized for photoacoustic imaging of deep brain tumours in living mice, as shown schematically in Figure 20.[188] Excellent image enhancement was observed

in vivo, aiding accurate tumour detection. It was found that deeper penetration was observed after 2 days compared with images produced 1 day after administration. The PDI nanoparticles are stabilized by amphiphilic molecules (DSPE-mPEG₅₀₀₀), shown in Figure 18. Together with other advantages such as high biocompatibility and photostability, it suggests that novel PDI-based nanoparticles have great potential for use as contrast agents.

3.7.3 Quantum Dots

As previously mentioned, quantum dots are commonly used for fluorescence imaging as they have high resistance to photobleaching compared with organic dyes. The most interesting property is that the absorbance properties can be altered by changing the size/shape and hence quantum dots can be engineered to attain absorbance in the NIR region. The limited quantum yield means that light is often converted into heat and hence quantum dots could be developed for PA imaging and PTT applications.[189]

3.7.4 Nanodiamonds

Nanodiamonds have excellent chemical inertness which is ideal for biomedical applications. They can be engineered and used as probes for PA imaging, however there are limited examples.[190] The ease of surface modification would allow for the conjugation of other NIR-active molecules to the nanodiamond surface

3.7.5 Polymer Nanoparticles

Semi-conducting polymer nanoparticles (SPN) contain polymers with π -conjugated backbones which are able to absorb light. SPNs with NIR absorbance have previously been used in solar cells, however more recently they have been investigated for their ability to provide PA contrast.[158] SPNs have various advantages such as high photostability, oxidative tolerance and a high molar extinction coefficient. A study was carried out to assess the contrast enhancement of SPNs compared to gold nanorods and it was found that the PA amplitude of SPNs was much higher than for gold nanorods of the same concentration, thus suggesting dose levels could be reduced.[191]

3.8 Multimodal Imaging

Some of the limitations of conventional bioimaging techniques could be circumvented by combining imaging modalities. This idea would be made simpler by designing novel contrast agents capable of providing contrast in multiple techniques. One example of this is where researchers have looked at making novel hybrid biomaterials that provide contrast for both MRI and PET.[94] By radioactively labelling a MRI contrast agent, the high sensitivity and good spatial resolution, of PET and MRI respectively, could be combined.

Another example is in brain tumour imaging where current techniques face obstacles, such as poor sensitivity and spatial resolution, leading to problems in locating the exact tumour location for efficient surgical resection. MRI has previously been used both pre-operatively and intra-operatively however inaccuracies may occur due to the short half-life of gadolinium(III) contrast agents in the blood. Combining MRI with other imaging techniques, such as photoacoustic and Raman imaging, could improve tumour delineation. Triple-modality nanoparticles were synthesized with a gold core (60 nm) surrounded by Raman active trans-1,2-bis(4-pyridyl)-ethylene protected by silica with gadolinium(III) chelate species attached to the exterior.[192] These novel nanoparticles showed superior relaxation enhancement ($3.0 \times 10^6 \text{ mM}^{-1} \text{ s}^{-1}$) for MRI, an extremely high optical absorbance coefficient compared with carbon nanotubes and low noise Raman signal for complete tumour resection. The results suggest that these triple-modality nanoparticles combine the complementary

properties from each imaging technique and, as they have an inert gold core, show high potential for clinical translation.

4 Biopolymers for Cell Encapsulation and Cell Delivery

In recent years, regenerative medicine therapies (RMTs) have been developed and intensively investigated for many applications including the treatment of some incurable diseases, regenerating organ and tissue loss due to disease and injury, and reducing reliance on transplantation.[163] RMTs can utilize stem cells although stromal cells and macrophages are also employed.[163] Stem cells have two distinct properties: the capacity to self-renew and the ability to differentiate into specialized cells. They are typically defined in terms of their potency, with unipotent stem cells giving rise to just one type of specialized cell; multipotent stem cells giving rise to a range of different specialized cells; and pluripotent stem cells generating all cell types within the mature organism. Progenitor cells, which are usually derived from stem cells, can also differentiate to generate different types of specialized cells but have a limited capacity to self-renew.[193] For RMTs, in addition to ethical issues (particularly relating to the use of embryonic stem cells), there are significant safety concerns associated with tumourigenicity and immunogenicity.[163, 194] Furthermore, understanding the biodistribution and fate of cells following implantation is very important for assessing safety and efficacy and also for understanding the mode of action.[163] The studies for this purpose are mainly performed by non-invasive *in vivo* imaging techniques, particularly those techniques with high penetration depth, high resolution, and the potential for whole body imaging. To address the safety issues faced by RMTs, strategies for cell encapsulation and cell delivery have been developed.

4.1 The Need for Cell Encapsulation and Hydrogels

Cell encapsulation has been frequently applied when transplanting therapeutic cells, in order to bring about several benefits. These mainly include: (i) addressing the immunogenicity when transplanting allogeneic cells, as the capsules act as an immune-isolating layer to prevent attack from immune cells; (ii) acting as a scaffold with suitable mechanical and chemical cues to support the growth of stem cells and regulate the differentiation of stem cells; (iii) serving as a barrier to avoid or reduce the leaking of therapeutic cells from the transplantation site.

Hydrogels are one of the most common ways to encapsulate cells (other methods may include semi-permeable membrane, etc.). Hydrogels are swollen polymer networks, held together by either chemical or physical crosslinking, which can retain a large amount of water.[195] The use of hydrogels took off in the 1960s, from simply forming hydrogels by polymerizing water-soluble monomers/polymers and smart hydrogels with various triggers, to stereocomplexed hydrogels and hydrogels from self-assembly of peptides/polypeptides.[195] When selecting hydrogels for cell culture, mechanical, structural, and compositional cues are often considered because they can regulate cell behavior, either alone or synergistically.[196] These cues are reflected by the hydrogel properties such as type of polymer, stiffness, porosity, and biodegradability.[197] In general, hydrogels are formed from natural polymers (e.g., fibrin, collagen, gelatin, polysaccharides) and synthetic polymers (e.g., polyacrylamides, poly(acrylic acid), poly(ethylene glycol), polyoxazolines, poly(vinyl alcohol), poly(lactide-co-glycolide), polycaprolactone).[197] There are commercial hydrogels which can be purchased from providers and used for cell culture directly. The most

commonly used hydrogels are prepared from collagen, fibrin, alginate, polyacrylamide, poly(ethylene glycol), hyaluronic acid, and polypeptides. For each type of hydrogel, different manufacturers may give different names.[198]

4.2 The Basics of Cell Encapsulation

Cell encapsulation may be classified into two categories. The first category is encapsulating cell in biodegradable hydrogels for tissue engineering.[199] This is in contrast to seeding cells in prefabricated porous scaffolds or hydrogels. To achieve cell encapsulation, cells are suspended in a liquid precursor and the solution is injected into the site of interest; *in situ* gelation of the solution ensures encapsulation. Due to the presence of cells, the solution components should be nontoxic and the gelation conditions should be mild so that the cells can survive and are not negatively impacted upon. For the hydrogels in cell encapsulation, small molecular monomers are not often used due to cytotoxicity concerns. Instead, macromers with molecular weight of 3 kDa or higher and natural polymers are generally used. The biodegradation rate of these hydrogels may be adjusted by choosing suitable precursor or incorporating hydrolytically or enzymatically labile segments during gelation.[199]

The second category of cell encapsulation, probably the more commonly referred to definition, is encapsulating cells for cell transplant, with the aim of drug delivery and/or cell delivery applications.[200, 201]. Implanting cells from allogeneic sources stimulates a response from the body's innate and adaptive immune systems. The primary goal of cell encapsulation is to suppress the attack by the immune system whilst maintaining controlled delivery of therapeutic products by the cells.[202] This means that the encapsulated cells should be able to grow well and perform the required functions. In addition to protecting the cells, the hydrogel should support the cell and allow two-way diffusion of nutrients and wastes/therapeutic agents.

The discussion here is focused on the second category of cell encapsulation although much of the discussion may be also valid for the first category of cell encapsulation.

While the first concept of cell encapsulation was mentioned in 1933, it is generally regarded that the first attempt of an 'artificial cell' by Chang in 1964 intensified the studies on cell encapsulation (Figure 19).[203, 204] In Chang's work, carbonic anhydrase was encapsulated in semipermeable microcapsules, which showed efficient catalytic hydration of CO₂ as the enzyme in free solutions and the activity remained after weeks of storage.[204]

For the encapsulated cells to survive, nutrients and oxygen should be able to sufficiently move across the hydrogel membrane while antibodies and immune cells are kept out. Therapeutic agents are excreted by the cells *via* diffusion out of the hydrogel membrane to achieve the targets of cell therapy.[205] Sufficient supply of oxygen is critical for the survival and healthy growth of the cells. This can be enhanced by the use of pre-vascularized support system and more discussion regarding this is given in the section below.

4.3 Important Parameters of Cell Encapsulation

4.3.1 Encapsulation Materials

The criteria which must be considered for selecting an encapsulation material include biocompatibility, immune-isolation, and capability of providing a suitable microenvironment that minimizes stress on encapsulated cells.[206] Rejection pathways should also be considered in order to find suitable encapsulation materials.[202] Different types of natural polymers have been used to encapsulate cells due to their intrinsic biocompatibility and biodegradability.[207] These may include DNA-based hydrogels, protein-based hydrogels (also peptide hydrogels) and polysaccharide-based hydrogels (alginate, chitosan, chondroitin sulfate, hyaluronic acid, agarose, xanthan).[199, 207] The hydrogels are formed by physical crosslinking (*via* hydrophobic interaction and ionic interaction), free radical polymerization, or chemical crosslinking of novel chemistry.[199, 207, 208] Some crosslinking methods that are particularly effective for injectable hydrogels are Michael addition (e.g., between thiols/amines and α,β -unsaturated carbonyl compounds), click reaction (Cu(I)-catalyzed reaction between azide and terminal acetylene groups), Schiff based reaction (between an amine and an aldehyde group), and photocrosslinked hydrogel (acrylate monomers or macromers by UV radiation).[208]

When preparing encapsulated cells for pre-clinical and clinical applications, simple physical crosslinking methods, *via* hydrophobic interaction (e.g., chitosan thermosetting gels) or ionic crosslinking (e.g., alginate crosslinked by Ca^{2+}) can be employed. For example, in a study to find the encapsulation system for pancreatic islets, a thousand combinations of polyanions and polycations were tested to form capsules simply by dropping each polyanion solution into each polycation solution.[209]

Alginate is the mostly used polymer for cell encapsulation; the majority of encapsulating gels are formed from sodium alginate (Alg) as polyanion and poly-L-lysine (PLL) as polycation.[202] Alginate is a natural anionic polymer composed of 1,4'-linked β -D-mannuronic acid (M) and α -L-gluconic acid (G) residues in different sequences (Figure 20).[207] The ratio of G to M blocks is an important parameter, which varies depending on its natural sources. It is generally believed that high G content contributes to a more rigid structure, whilst high M content in alginate is preferred for pliable gels.[207] There are contradictive findings about the G content of alginate for cell encapsulation although some researchers believed high G alginates are more suitable for cell encapsulation.[202]

Mechanical stability of the microcapsules is an important parameter. Stable microcapsules can protect the cells and are easy for handling. In general, mechanical stability may be improved by using a high concentration of precursors and high crosslinking density; however, this may not be applicable for a selected encapsulation system. In the case of alginate, this has been achieved by the use of different polycations, e.g., the use of poly-L-ornithine, Ba^{2+} , to form a layer-by-layer coating.[202]

4.3.2 Fabrication Methods

Cells can be encapsulated in bulky hydrogels, hydrogel beads, microcapsules and other micronized systems. For encapsulation in bulky hydrogels, cells are suspended in a liquid precursor solution, followed by gelation. The cell-encapsulated hydrogels can also be formed in hollow fibers to suit the need of different applications.[200] To form hydrogel beads in solution, often seen as alginate beads, alginate solution containing cells can be injected into aqueous CaCl_2 solution. When a polycation solution is used, capsules may be produced instead of beads, due to the limited mass transfer of polycation molecules.[209] The size of the beads may be reduced using a pressurized nozzle or vibration nozzle.[210]

One challenge is that bulky hydrogels and thick capsules can be a significant barrier for the diffusion of nutrients and oxygen, thus having a considerable effect on cell viability. When cells are encapsulated in microcapsules, the large surface-to-volume ratio facilitates transport of nutrients. For example, one study showed that cell viability and metabolic activity was optimal when the cells were encapsulated in microcapsules in the range of 100 μm . [211] Cell encapsulation in microcapsules or microspheres can be realised *via* an emulsion-based approach, where the droplets are turned into microspheres or microcapsules. [210, 212] However, there may be limitations for the emulsion method because of the use of organic solvent. Cell encapsulation can also be effectively fabricated by microtechnologies, including microfluidics and micromolding methods. [200]

4.3.3 Nutrients and Oxygen Transport

While the pores of the microcapsules are small enough to keep out immune cells, they should be large enough to allow sufficient transport of nutrients, oxygen and therapeutic agents. In addition to shortening the diffusion path, increasing the porosity is a way to promote transport.

One of the big challenges is to supply sufficient oxygen to maintain the viability and function of the encapsulated cells. [213] The oxygen transport may be enhanced by (i) using a smaller device or microcapsule to reduce diffusion distance; (ii) using organic compounds with high oxygen solubility (such as perfluorocarbons, silicone oils, soybean oils) in the microcapsules or in the device to increase the permeability of oxygen; (iii) inducing neovascularization adjacent to the microcapsules or utilizing pre-vascularized support (Figure 22b) to bring blood flow close to the tissue; (iv) provision of exogenous oxygen (gaseous oxygen supply, *in situ* oxygen generation) to increase partial pressure of oxygen adjacent to the tissue. [213]

4.4 Applications of Cell Encapsulation

Cell encapsulation has been used for drug delivery and cell delivery. [201, 205] There has been great interest in encapsulating pancreatic islet cells to treat diabetes [206, 210, 212] and also various cancers, [201, 205] as well as diseases that affect the brain [214] and kidney. [215] More examples are given in Table 2. [202]

There is no definite distinction between drug delivery and cell delivery *via* cell encapsulation. In general, biopharmaceutical drug-loaded cells may be used for drug delivery while the delivery of therapeutic agents from engineered cells or stem cells may be regarded as cell delivery. [201, 216] The use of cell encapsulation for delivery of therapeutic agents has two advantages: (i) the immune-isolating microcapsules make it unnecessary to take immunosuppressant drugs and (ii) long-term delivery may be achieved, depending on the types of cells used (Figure 21). [216]

The selection of suitable cells for encapsulation is essential for biomedical applications. Many cell lines have been used, some of which are given in Table 2. The proliferation behavior of the cells should be considered as with prolonged growth and proliferation. The cells may eventually fill the capsular space, which may hinder diffusion of therapeutic agents. Cells that do not proliferate can provide an advantage for long-term delivery. [202]

For drug delivery by encapsulated cells, some types of cells are more widely used than others. [201] Red blood cells are used as drug carriers because they have a long circulation time, high drug-loading

capacity, and good biocompatibility. Macrophages are derived from monocytes; the ability of monocytes to migrate towards pathological sites of infection/inflammation/tumors, and then differentiate into macrophages, has been exploited for drug delivery. Dendritic cells are antigen-presenting cells known to interact with T and B lymphocytes as well as natural killer cells. Vaccination with dendritic cells has been used as a new cancer treatment method.[201] This practice may be enhanced by incorporating drug-releasing and cytokine-releasing entities into the hydrogel capsules during the fabrication stage.[206]

For the delivery of therapeutic molecules secreted by engineered cells, the use of bacteria-based delivery vehicles has made good progress, as applied to cancer treatments. Anticancer agents including cytotoxic agents, cytokines, tumor antigens and antibodies have been delivered by bacteria. The bacteria can migrate to tumor cells (partially dependent on oxygen level) and penetrate into cancer tissue upon arrival at the site.[201]

A fast growing field is the encapsulation of stem cells for regenerative medicine, where mesenchymal stromal/stem cells (MSCs) are most commonly used in the investigations.[201, 206, 217] Unmodified MSCs can release factors that inhibit the proliferation of several tumor types.[218] The homing capacity of MSCs has been exploited for tumour-selective migration and release of secreted factors.[219] Some other examples include implanting encapsulated MSCs to accelerate bone regeneration or induce vascularization.[216]

For stem-cell based therapy, the capsules act more as a scaffold to promote the viability, proliferation and differentiation of the stem cells, rather than as an immunobarrier.[216] As such, the rate of degradation of the capsule is a critical factor, as it will allow space for cell proliferation, cell migration, and the release and diffusion of therapeutic agents.[216] Monitoring the status of the hydrogels, using imaging techniques, can provide key information on cell encapsulation/migration and therapeutic efficacy. This can be achieved by functionalizing the hydrogels with fluorescent groups or encapsulating NIR-active molecules.[220, 221]

Monitoring the fate of the cells, capsules/hydrogels, and the surrounding microenvironment *in vivo* after implantation is critical for the understanding and developing of RMTs. This can be achieved by non-invasively imaging live cells and monitoring the stability/degradation of the hydrogel in order to obtain data/information on cell viability, biodistribution, tumorigenicity, immunogenicity, cell fate, and eventual therapeutic efficacy.[127, 163, 221, 222] Many of the applicable imaging techniques have been briefly described in the previous sections. Advances in instrument technologies combined with developing new imaging probes (depending on the types of the instruments), with excellent biocompatibility, high intensity and high stability, are crucial to enhance the understanding at the macroscopic, microscopic, and molecular levels.

5 Conclusions

Biomaterials, including biopolymers and nanostructured materials, have been used for poorly water-soluble drug nanoparticles, bioimaging, and cell encapsulation. In the efforts to address poor water-solubility and poor bioavailability of drug compounds, various techniques, categorized into top-down and bottom-up approaches, have been developed to fabricate drug nanoparticles. The improvement in water solubility and bioavailability is attributed to the enhanced dissolution rate and saturation solubility due to the high surface of drug nanoparticles. Furthermore, aqueous drug nanoparticle dispersions may be administered intravenously or *via* respiratory delivery. This is facilitated by the

transport of small drug nanoparticles. Biocompatible polymers and surfactants are used in these processes with the main role being to stabilize the drug nanoparticles during preparation and storage, in solid state and in liquid suspension. There is also the potential to modify drug nanoparticles with suitable ligands in order to achieve targeted and smart delivery.

Bioimaging techniques are essential for diagnosis and treatment of disease and are available both pre-clinically and clinically. Some of the commonly used and newly developed bioimaging techniques have been discussed in this chapter. Magnetic resonance imaging is one of the more attractive techniques, compared with computed tomography and positron emission tomography scans, as it does not utilise ionising radiation. Ultrasound is also a safer option however it relies on movement within the body, whereas many optical imaging techniques such as fluorescence imaging suffer from poor spatial resolution and hence have limited clinical use. Photoacoustic imaging is an attractive technique which is not yet widely used, however it is being trialled for different applications and has exceptionally high potential, as it has been shown to have excellent spatial resolution with a relatively high imaging depth. For all these imaging techniques, a very important aspect of research is to develop biocompatible contrast agents to enhance imaging resolution and/or penetration depth. Each of these bioimaging techniques has their own advantages and disadvantages, depending on cost, convenience, imaging time, resolution, penetration depth, and potential side effects. Suitable imaging techniques may be selected based on the needs of the patient. An exciting development is the combination of multiple imaging methods with the use of contrast agents containing multiple imaging modalities. This can combine the advantages of different imaging techniques while hopefully avoiding the disadvantages at the same time.

Non-invasive *in vivo* bioimaging techniques are particularly attractive. They are essential for understanding the biodistribution, fate, and therapeutic efficacy of implanted or injected cells in regenerative medicine therapies. Stem cells and progenitor cells are commonly used in regenerative medicine, however in order to address the safety issues and improve therapeutic efficacy, cell encapsulation and cell delivery have been widely employed for regenerative medicine. Biopolymer hydrogels are essential for encapsulating cells. After introducing the need and basic principles of cell encapsulation, different encapsulation parameters/methods are discussed. The applications of cell encapsulation for regenerative medicine are subsequently described.

With the applications discussed above, the use of biopolymers and associated nanostructures is necessary. For biomedical applications, biocompatibility, biodegradation rates/routes, and clearance from the body must be considered together. There are two main roles of biopolymers which have been identified for these applications. The first role is that the biopolymers act as stabilizers (to form nanoparticles and during freeze-drying process), where suitable functional groups and/or amphiphilic properties are important. The second role is to form hydrogels for the encapsulation of cells. In relation to the types of cells encapsulated, the mechanical property (stiffness, crosslinking density), surface group, and the morphology of the hydrogels are important parameters. For future development in biopolymers, two important routes may be proposed. The first route is to modify the currently approved or used polymers with new functional groups to improve the current properties or achieve new properties. The second route is *via* the design and synthesis of novel biopolymers, which should exhibit the attributes required for targeted applications.

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Figures and Table

Figures:

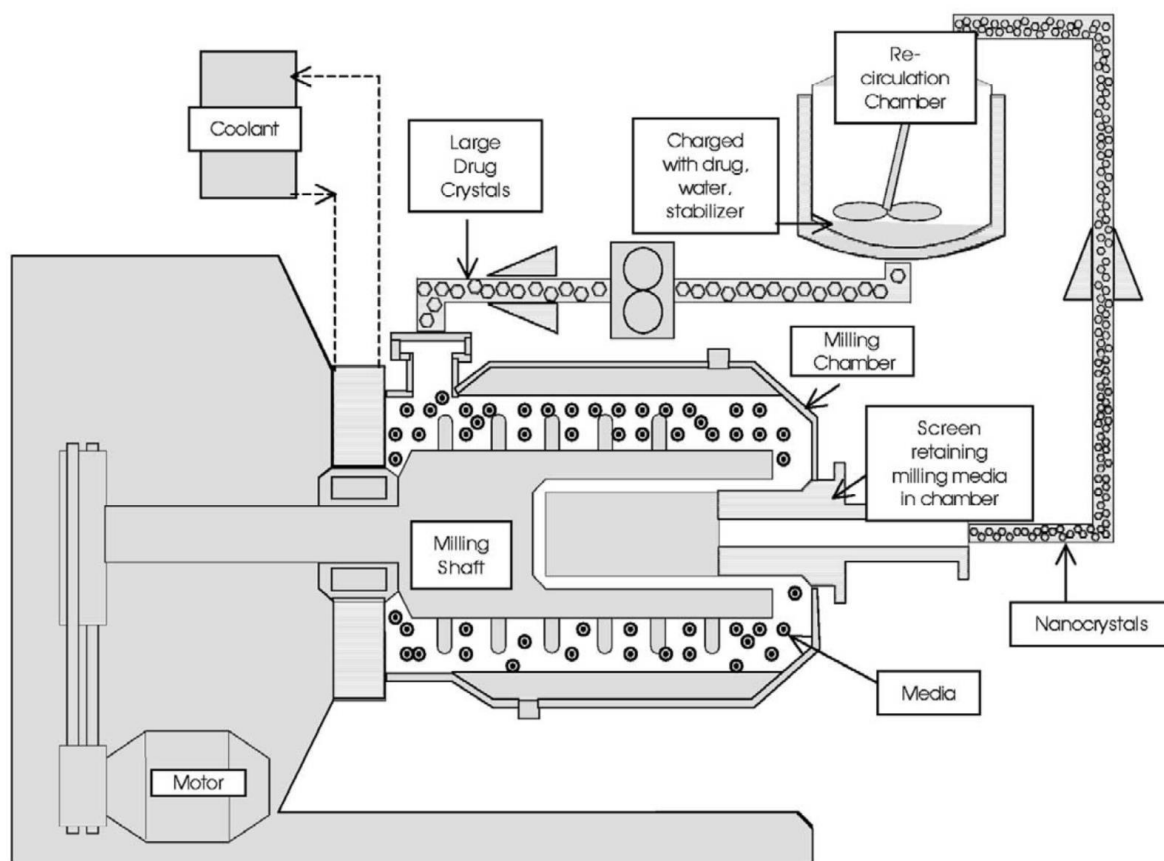


Figure 1. A schematic representation of the wet media milling process. Reprinted with permission from ref 21

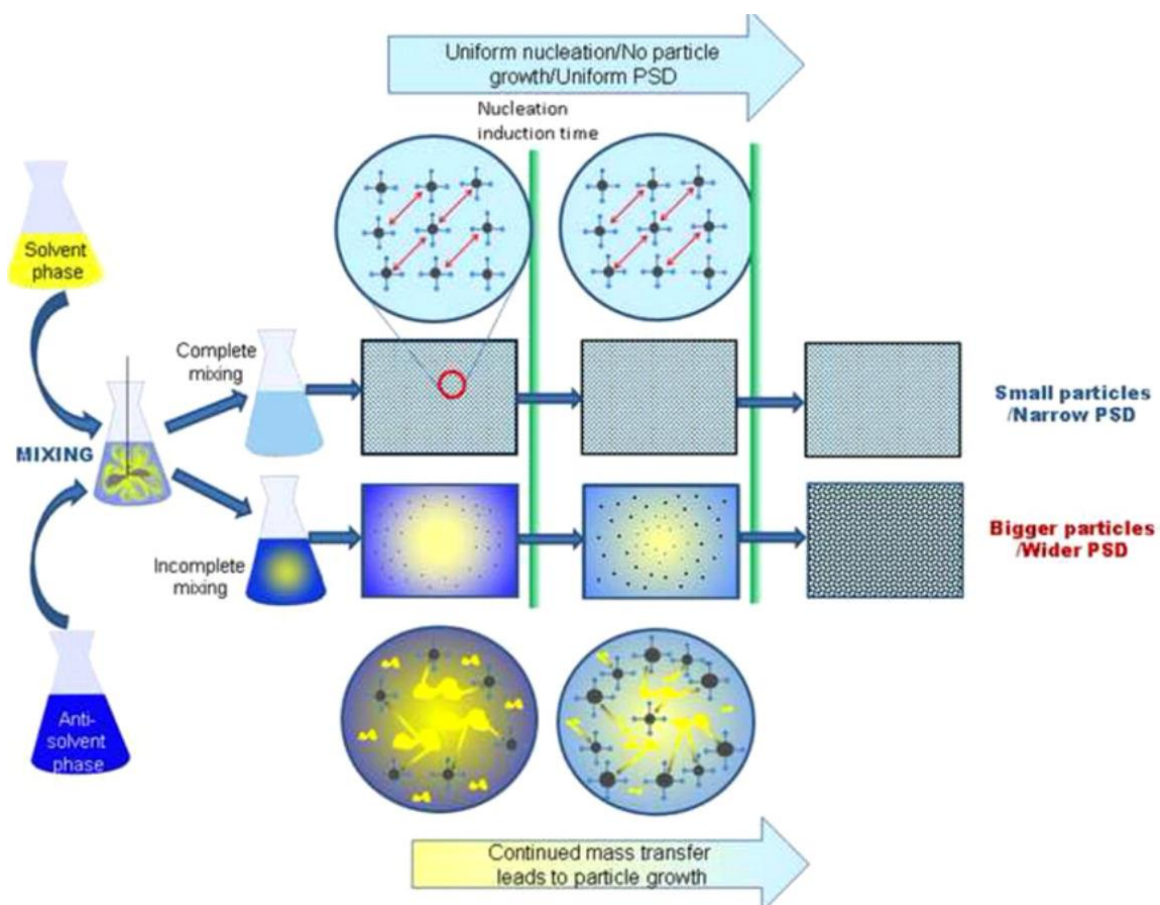


Figure 2. The effect of mixing on nanoprecipitation. Reprinted with permission from ref 37.

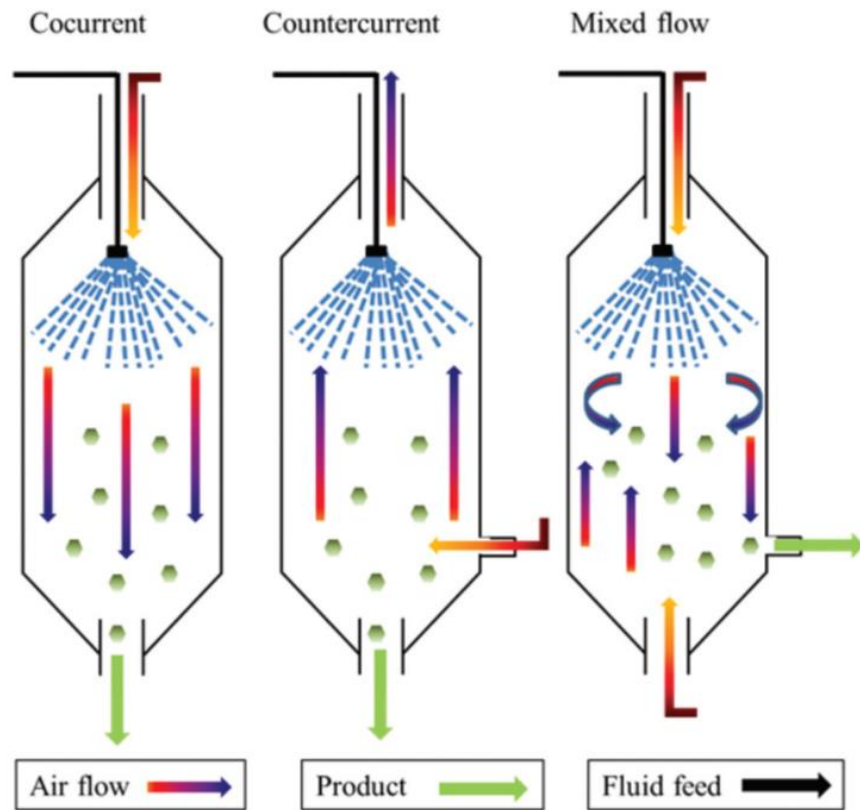


Figure 3. Schematic representation of the possible hot air flow directions in the spray-drying process. Reprinted with permission from ref 45.

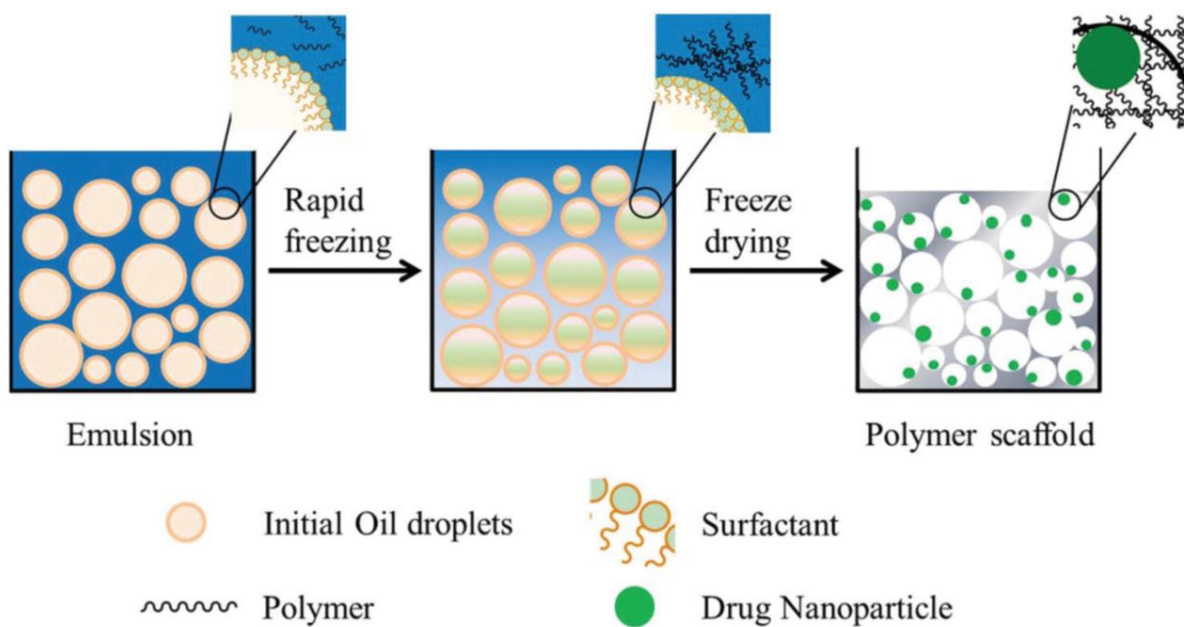


Figure 4. Schematic representation of the emulsion freeze-drying process to form drug nanoparticles. Reprint with permission from ref 45.

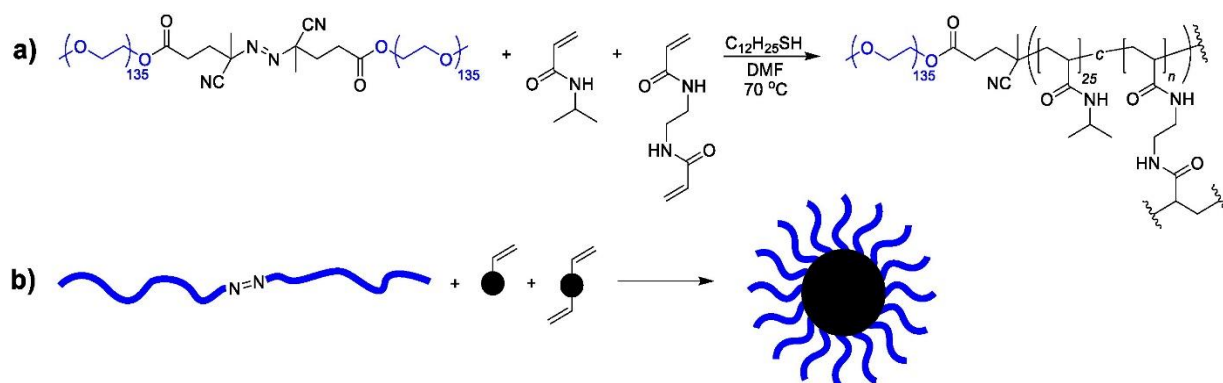


Figure 5. A schematic representation of (a) the synthetic route and (b) a cartoon representation of the synthetic route for preparation of lightly crosslinked branched copolymer PEG-PNIPAM. Reprinted with permission from ref 58.

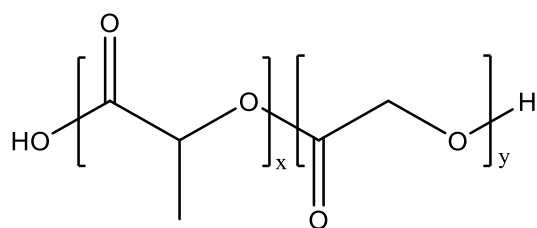


Figure 6. Chemical structure of PLGA where x and y are the number of repeating units of lactic acid and glycolic acid respectively.

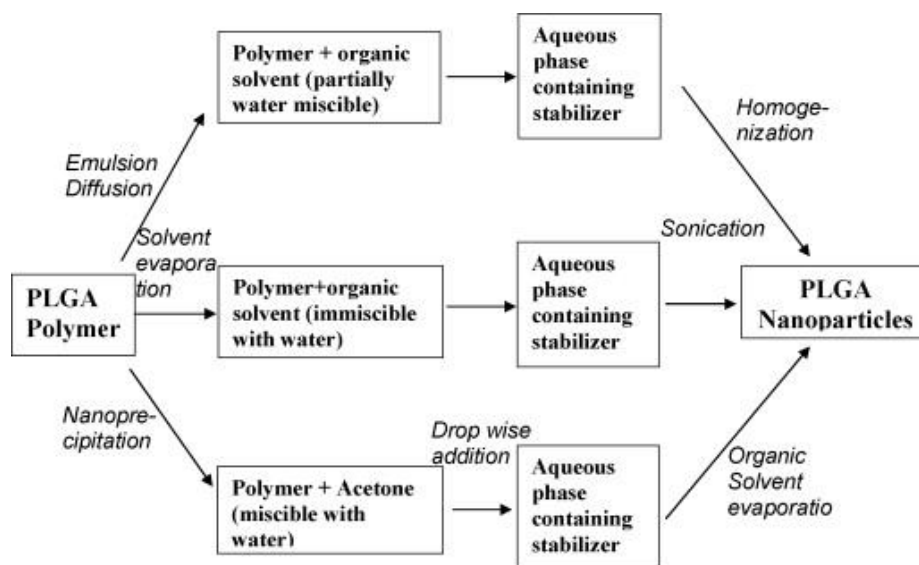


Figure 7. Summary of techniques that can be used to synthesized PLGA nanoparticles. Reprinted with permission from ref 63.

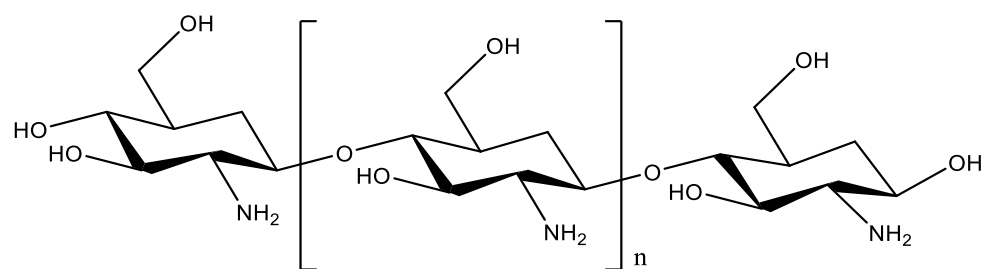


Figure 8. Chemical formula of Chitosan.

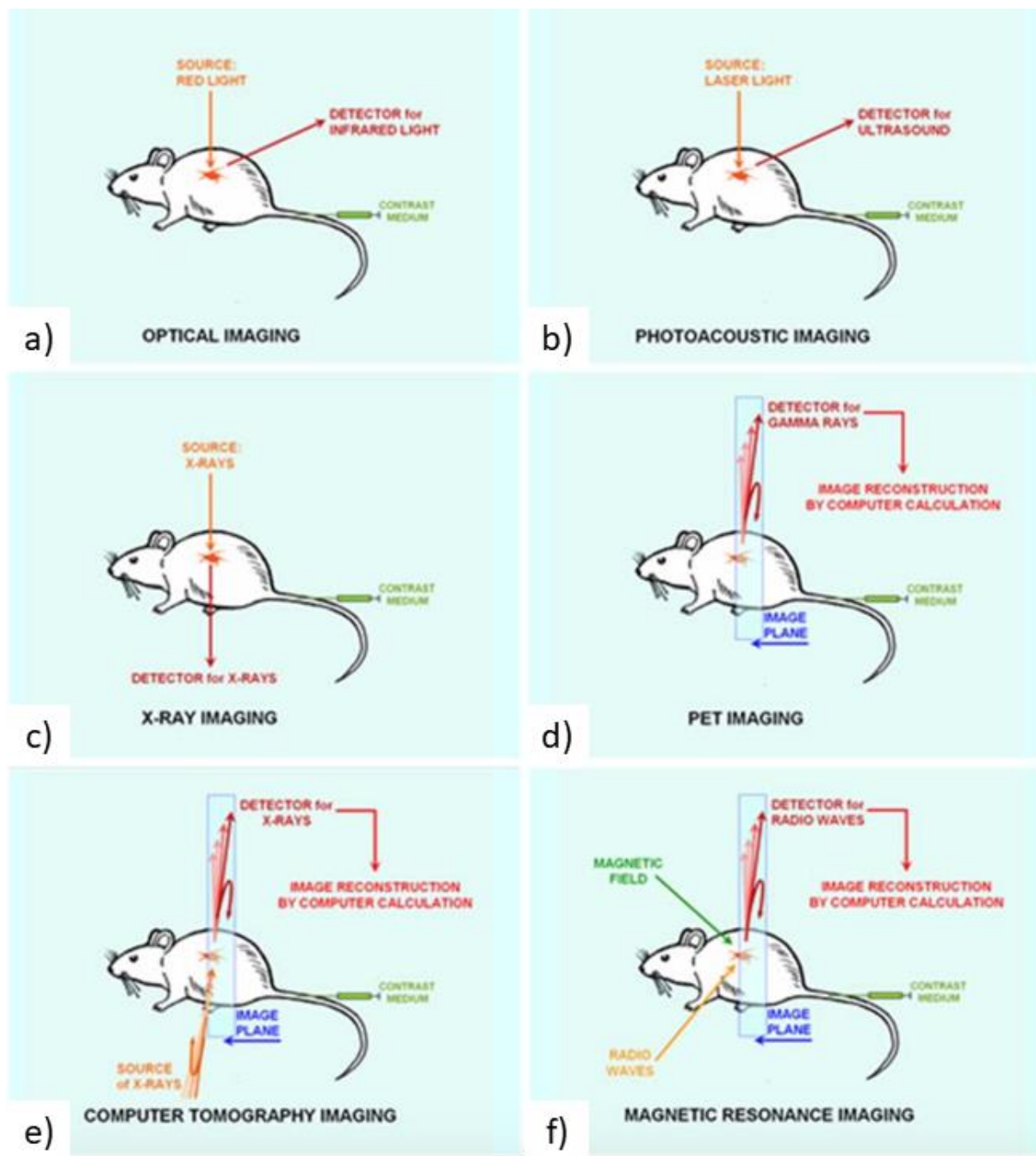


Figure 9. Visual representations of the principles behind different imaging modalities. Reprinted with permission from ref 78.

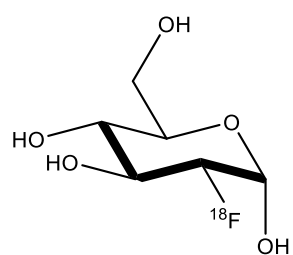


Figure 10. Chemical structure of Fludeoxyglucose.

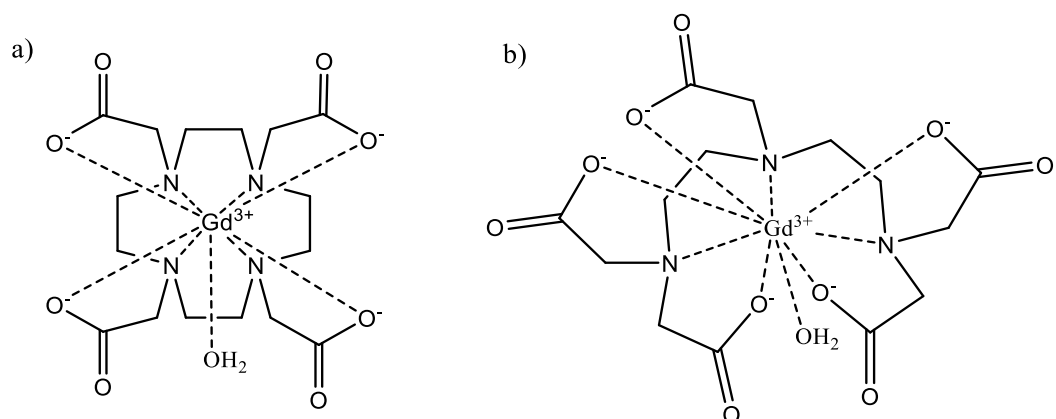


Figure 11. Chemical structure of (a) Dotarem® contrast agent which shows Gd(III) chelated by 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) and (b) Magnevist® which contains diethylenetriaminepentaacetic acid (DTPA).

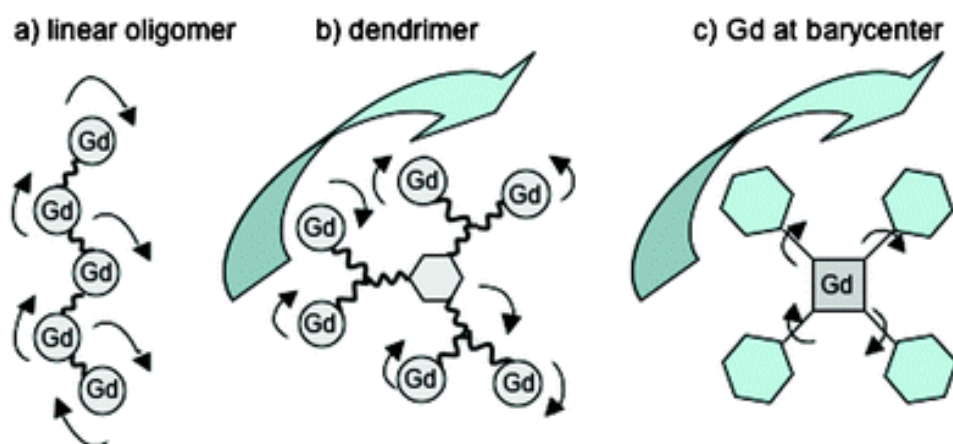


Figure 12. Different ways of increasing the relaxivity of gadolinium chelate species where constructs can be (a) linear oligomers, (b) dendrimers or (c) with gadolinium at the barycentre. Reprinted with permission from ref 105.

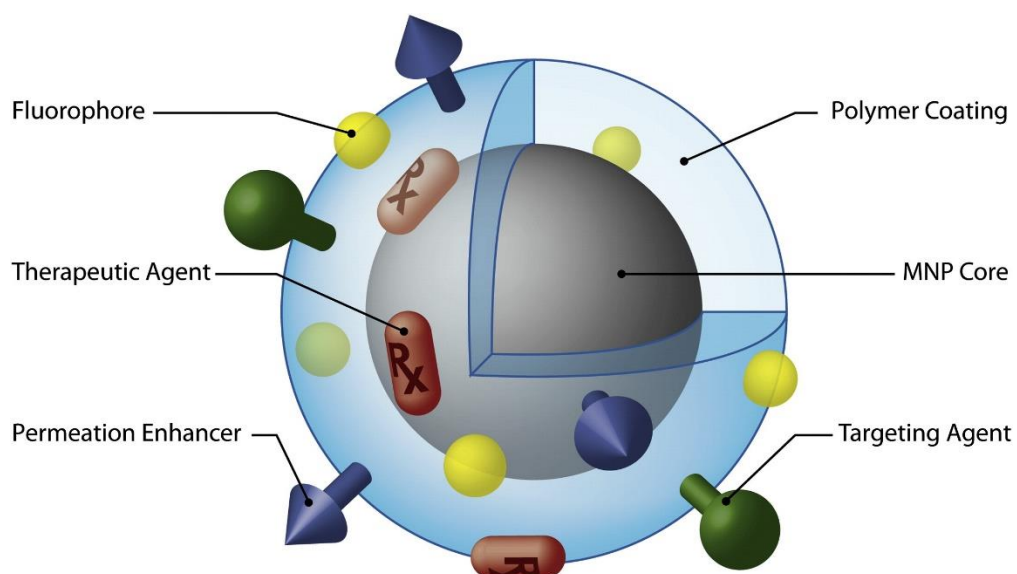


Figure 13. Examples of different functional ligands which can be incorporated onto core-shell magnetic nanoparticles (MNP). Reprinted with permission from ref 122.

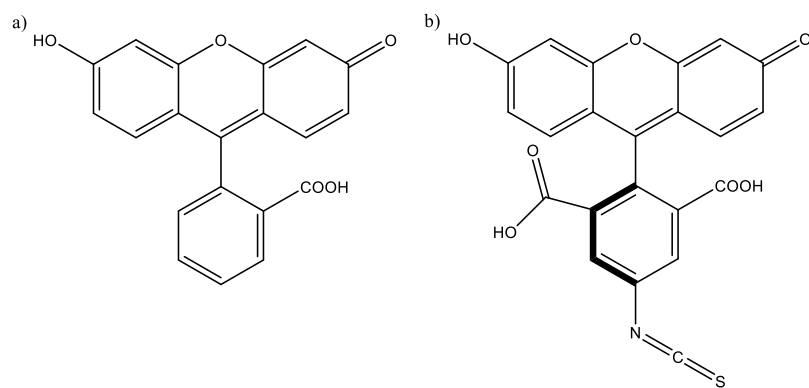


Figure 14. Chemical structure of (a) fluorescein and (b) fluorescein isothiocyanate.

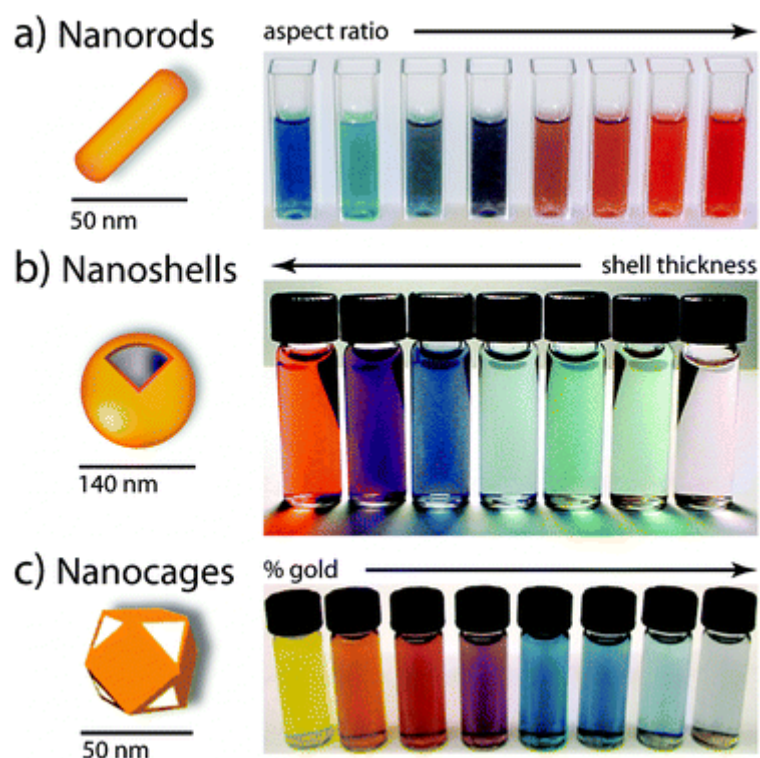


Figure 15. Visual representation of how absorbance properties can change upon modification of nanoparticle properties for (a) gold nanorods, (b) silica-gold core shell nanoparticles and (c) gold nanocages. The wavelength of absorption is dependent on physical parameters such as the aspect ratio, shell thickness or galvanic displacement by gold. Reprinted with permission from ref 168.

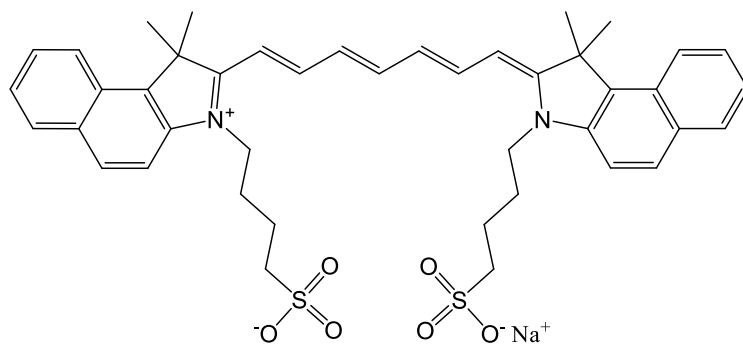


Figure 16. Chemical structure of indocyanine green.

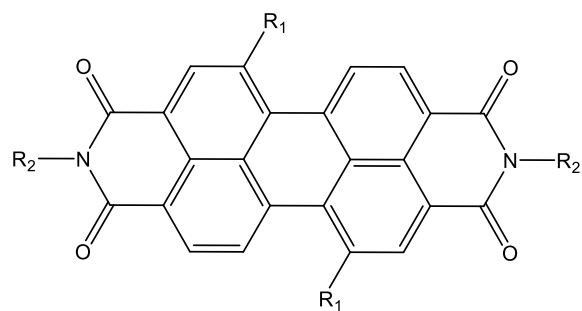


Figure 17. General chemical structure of perylene diimide derivatives.

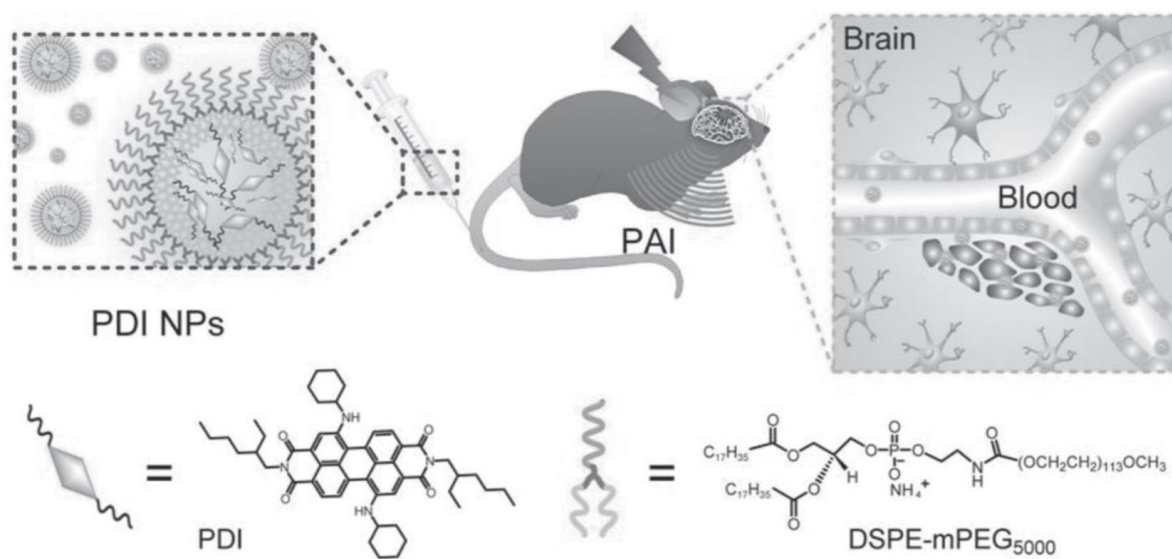


Figure 18 Visual representation of PDI nanoparticles made with DSPE-mPEG₅₀₀₀, and their injection into a mouse. Reprinted with permission from ref 188.

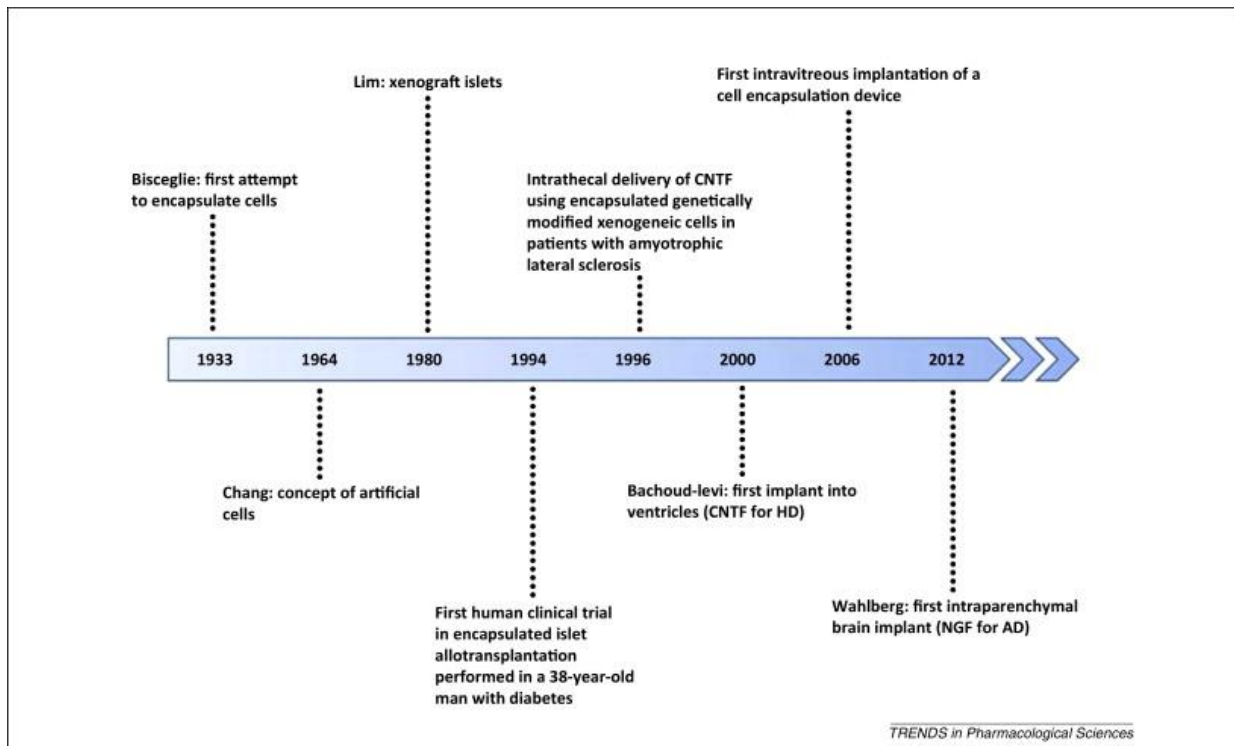


Figure 19. Timeline showing the milestones of cell encapsulation progress. Reprinted from Ref 203 with permission.

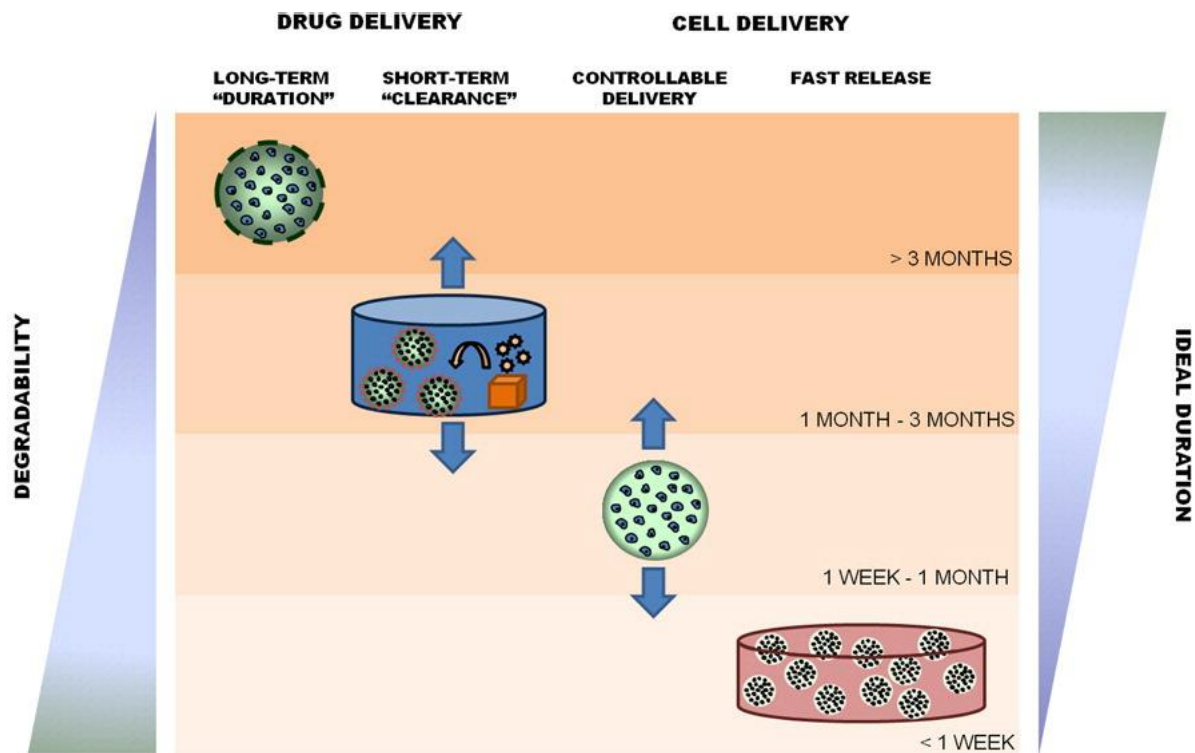


Figure 21. Represented timescale of drug delivery and cell delivery *via* cell encapsulation Reprinted from Ref 216 with permission.

Table 1. Polymer stabilizers used for preparation of poorly water soluble drug nanoparticles

Polymer stabilizer	method	Drug nanoparticles	references
poly(vinylpyrrolidone/vinyl acetate) (Kolidon VA64®)	Dry milling	Probucol	18
Hydroxypropyl cellulose	Ball milling	Naproxen	25
Amino acids	Ball milling	indomethacin	26
Tween 80/Poloxamer 188	High pressure homogenization	amphotericin	32,33
Cremophor EL	High pressure homogenization	Paclitaxel, docetaxel	33, 34
Poloxamer 188	High pressure homogenization	Piroxicam	35
Poloxamer 188	nanoprecipitation	Paclitaxel encapsulated in PLGA	41
Poly(vinyl alcohol)	Supercritical fluid precipitation	ibuprofen	44
Poly(caprolactone)	Spray drying	Indomethacin encapsulation	48
Poly(vinyl alcohol), Polyvinylpyrrolidone, Pluronic, Tocopherol polyethylene glycol succinate	Emulsion freeze drying	Several poorly water soluble drugs	53, 57
Poly(ethylene glycol)-b-(N-isopropylacrylamide)	Emulsion freeze drying	Indomethacin, ibuprofen, ketoprofen	58,59

Table 2. Different cell sources used for cell encapsulation (adapted from ref 202).

Cell type	Application	Material
Fibroblasts	Metabolic deficiencies, neurotrophic factors, epilepsy	Alginate, HEMA-MMA
Myoblasts	Metabolic deficiencies, neurotrophic factors, cancer	Alginate, HEMA-MMA
Kidney cells	Hemophilia, neurotrophic factors, antiangiogenesis	Alginate
Pancreatic islets	Diabetes	AN69, Cellulose sulfate
Ovary cells	Fabry disease	Alginate, HEMA-MMA
Parathyroid cells	Artificial organs	Alginate
Hepatocytes	Liver transplantation	Alginate, HEMA-MMA
Chondrocytes	Bone and cartilage regeneration	Alginate
Leydig cells	Hormone replacement	Alginate
Adrenal chromaffin cells	Parkinson's disease, chronic pain	Alginate
Stem cells	Bone regeneration	Alginate
Pheochromocytoma cells	Neurotrophic factors, neurotransmitters	Alginate, HEMA-MMA
Myeloma cells	Hepatic growth factor	Alginate
Hybridoma cells	Antibody production	Alginate, acetate, alginate-agarose
Tumor cells	Cancer vaccine, interleukins	Alginate, chitosan
Virus producer cells	Cancer	Cellulose sulfate
Bacteria	Elimination of urea	Alginate